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ARTIFICIAL CELLS, BLOOD SUBSTITUTES, AND IMMOBILIZATION BIOTECHNOLOGY

November 1994

Aims and Scope. This journal covers artificial cells, blood substitutes, and immobilization biotechnology. The emphasis of this journal is to allow for interdisciplinary interactions. Therefore, we welcome approaches based on biotechnology, chemical engineering, medicine, surgery, biomedical engineering, basic medical sciences, chemistry and others. The following areas are particularly welcomed.

1. Immobilized bioreactants including cells culture, microorganisms, enzymes, drugs, receptors, sorbents, immunosorbents and other biologically active molecules.
2. Artificial cells, microcapsules, liposomes, nanoparticles and other carriers.
3. Blood substitutes from fluorocarbon, modified hemoglobin, encapsulated hemoglobin, synthetic heme, recombinant hemoglobin, and others. Chemistry, methods, in-vitro studies, in-vivo evaluations and clinical results.
4. Microencapsulation and other methods of immobilization of cells (e.g. hybridoma, endocrine cells and liver cells, etc.) or microorganisms. Cells immobilized by different approaches. Methods, evaluation, and applications. Cell culture technologies related to immobilization. Hybrid artificial organs based on cell cultures.
5. Enzyme replacement, enzyme therapy, immunosorption, detoxification, hemoperfusion, metabolite conversions and drug delivery.
6. Design, evaluation and clinical application of hemoperfusion, artificial kidneys, plasmapheresis, and other artificial replacements.
7. Synthetic and biological biomaterials related to artificial cells and immobilization biotechnology. Blood compatible materials. Synthesis, biocompatibility, blood compatibility and evaluations.
8. Biotechnologically derived biologically active molecules related to artificial cells and immobilization biotechnology.
9. Drug delivery systems.
10. Other related areas including new approaches using biotechnology, computer, and other novel high technology.

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EDITORIAL

ARTIFICIAL CELL INCLUDING BLOOD SUBSTITUTE AND BIOMICRO-ENCAPSULATION : FROM IDEAS TO APPLICATIONS

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How long does it take for ideas and basic research to germinate into practical applications? It seems to depend on public demand and need. It also depends on the availability of related technology. Artificial cells including blood substitutes and biomicroencapsulation are good examples of this.

The first artificial cells were prepared and reported by Chang in 1957 and 1964 (1). "Artificial Cell ..... is an idea involving the preparation of artificial structures .... for possible replacement or supplement of deficient cell functions. It is clear that different approaches can be used to demonstrate this idea."(1972)(1)

In the 1960's there was a severe shortage of dialysers for use in medicine. Our first report of basic research using artificial cells in hemoperfusion was in 1966 (2). Public demands prompted us to develope this idea into a practical system by 1969 (2) and Phase I safety clinical trial by 1970 (2). Our Phase II efficacy clinical trials followed quickly: renal failure in 1971(2), liver failure in 1972 (2) and acute poisoning in 1973(2). This has been a routine clinical proceedure around the world since that time(2). Unfortunately, the many other areas of artificial cells had to wait for many years before there were public demands and availability of related technology for developing them for applications. Let us look at some examples.

I. MODIFIED HEMOGLOBIN BLOOD SUBSTITUTES:

Artificial red blood cells were first reported in 1957 in the form of encapsulated hemoglobin(1). Crosslinked hemoglobin was first reported in 1964 (1).

Unfortunately there was no public demand at that time. It is the recent public concern of H.I.V. in donor blood that stimulates major research & development in this area in the last 7 years(3).

A. Crosslinked hemoglobin:

In 1964 we used a bifunctional agent to prepare modified hemoglobin blood substitutes (1). This is to crosslink hemoglobin to form polyhemoglobin either as a membrane or as aggregates. The reaction is as followed:

$$\underset{\text{bifunctional agent}}{\mathbf{Cl\text{-}CO\text{-}(CH_2)_8\text{-}CO\text{-}Cl}} + \underset{\text{Hemoglobin}}{HB\text{-}NH_2}$$

$$= HB\text{-}NH\text{-}\mathbf{CO\text{-}(CH_2)_8\text{-}CO}\text{-}NH\text{-}HB$$

Crosslinked hemoglobin (Chang 1964)

Thrity years later, the modern approaches also make use of bifunctional agents. These are used to crosslink hemoglobin to form polyhemoglobin, conjugated hemoglobin or intramolelcularly crosslinked hemoglobin. These bifunctional agents include dialdehydes like glutaraldehyde, open ring sugars and many others (3). One example is the use of a bifunctional agent, glutaraldehyde, to crosslinked hemoglobin into polyhemoglobin. Gould's group is using this polyhemoglobin in their Phase II clinical trial(3). The reaction is as follows:

$$\underset{\text{bifunctional agent}}{\mathbf{H\text{-}CO\text{-}(CH_2)_3\text{-}CO\text{-}H}} + \underset{\text{Hemoglobin}}{HB\text{-}NH_2}$$

$$= HB\text{-}NH\text{-}\mathbf{CO\text{-}(CH_2)_3\text{-}CO}\text{-}NH\text{-}HB$$

Crosslinked hemoglobin (Gould et al 1994 Phase II Clinical Trial)

This 1994 modern approach bears a striking resemblance to the original idea of 1964. Why is it then that crosslinked hemoglobin has not been developed for clinical use until more recently? Public demand for blood substitute because of H.I.V. is very recent. This leads to extensive studies by many groups resulting in much additional progress and new technology in this area. Areas investigated include new crosslinking agents, intramolecular crosslinkage, conjugated hemoglobin, new modifiers, bovine hemoglobin, hemoglobin from recombinant technology, transgenic hemoglobin, physiopathology and clinical trials (3).

The following groups have contributed extensively to progress in this area. These include Abuchowski, Agishi, Bakker, Benesch, Bund & Jandl, Biro, Bucci, Chang, DeVenuto, Estep, Faivre, Feola, Fratantoni, Gould, Greenburg, Hedlund, Hess, Hori, Hsia, Iwashita, Jesch, Keipert, Klugger, Magnin, Manning, Messmer, Moss, Nose, Pristoupil, Pluira, Segal, Sekiguchi, Sideman, Shorr, Valeri, Winslow, Wong and many others (3). They have investigated in depth the best types of crosslinking agents. They have also improved the oxygen carrying and release functions of crosslinked hemoglobin by suitable uses of crosslinkers or modifiers. Furthermore, there is now much basic information to allow investigators to avoid problems related to toxicity in hemoglobin preparations. Ultrapure hemoglobin were reported by Winslow's group, this was improved further by Pluira et al (3).

An earlier problem was that animal safety results could not be applied directly to human. A number of approaches have been studied to solve this problem. For example, Chang & Lister have devise an in vitro screening test for this(3). This is done by adding modified hemoglobin to human plasma in a test tube, and test for complement activation. This is one of the ways to help bridge the gap between animal safety studies and clinical trials in human(3). A number of centres are completing their Phase I clinical trial (3). Gould et al in 1994 has started their Phase II clinical trial(3). Rapid progress in the area of crosslinked hemoglobin as blood substitute is shown by the many papers in this congress.

B. Artificial red blood cells from encapsulated hemoglobin

The first artificial cell is in the form of artificial red blood cells (arbc) containing hemoglobin (Chang 1957)(1). This has acceptable P50 for oxygen release. We found that artificial red blood cells larger than 1 micron diameter were removed from the pulmonary circulation(1). Those 1 micron diameter or smaller were removed by the reticuloendothelial system. We also found that removal of sialic acid from red blood cell (rbc) membrane resulted in rapid removal of rbc by the reticuloendothelial system (RES) (Chang 1965,72)(1), We therefore studied the effects of surface properties of arbc(1). This included the use of membranes with surface charge, crosslinked protein, bilayer lipid complexes, polysaccharides and others (1). Some of these changes improved the circulation time by decreasing RES uptake.. Circulation time was further improved when new technology allows the preparation of much smaller lipid membrane artificial red blood cells of 0.2 micron diameter (Djordjevich & Miller, 1980)(2). An increasing number of groups are working on this approach, especially after the problem of H.I.V.. The many groups in this area include: Hunt, Farmer, Tsuchida, Schmidt, Rudolph, Rabinovic, Chang, Mobed, Nishiyia, Usuba, Lee, Szebe, Sinohara, Kobayashi, Takahashi, Beissinger, Usuba and many others. Research includes variations in surface properties, new techniques in preparation and improvements in membrane strength. This results in increase in circulation half-time to about 1 day. Artificial red blood cells can now be used in acute situations like resuscitation of

hemorrhagic shock and exchange transfusion in animal studies. Other new approaches are in the horizon. Tsuchida in Japan incorporates synthetic heme into artificial cells(2). Chang & Yu (1992) has used biodegradable nanocapsules to encapsulate hemoglobin to form a more stable system (2). Many of the workers in this area are reporting their recent research in this congress.

BIOENCAPSULATED CELLS & MICROORGANISMS FOR CELL & GENE THERAPY & TISSUE ENGINEERING

As early as 1965 and 1966 Chang has used a drop technique for encapsulating intact cells inside artificial cells(1). At that time he proposed that:

"...protected from... immunological process... while permeable to small molecules.... encapsulated endocrine cells might survive and maintain an effective supply of hormone.. immunologically isolated"(1)

"For organ deficiency... cultures of liver cells... in artificial cells" (1)

However there was no public demand nor interest for this. This idea had to wait for years until the modern era of biotechnology. Since 1980, Sun's group has carried out extensive studies on the use of microencapsulated islets for diabetes mellitus(4). They show that islets inside artificial cells are indeed prevented from immunorejection after implantation into animals. They can indeed remain viable and can secrete insulin to control the glucose levels of diabetic rats. They also extend their studies to other endocrine tissues. Many other groups have developed this approach further(4). The most recent clinical trial by Soon-Shiong et al in 1994(4) shows that implantation of encapsulated islets can maintain a diabetic patient normoglycemic without the need for insulin injections.

Chang's group concentrates on artificial cells containing hepatocytes for liver support(5). In 1986 they first show that implantation increases the survival time of fulminant hepatic failure rats(5). They report in 1988 that xenografts of artificial cells containing rat hepatocytes are not immunorejected when transplanted into mice(5). Furthermore, they report in 1989 that implantation into Gunn rats effectively lowers the high bilirubin levels(5). Dixit's group later supports this finding and extends this to long term treatment in Gunn rats(5).

Many groups are carrying out extensive studies on artificial cells containing living cells (4-6). These include those of Sefton, Reach, Sun, Chang, Tice, Shiotani, Soon-Shiong, Dixit, and others (4-6). Many of them are reporting their recent results in this congress. For permanent long term implantation of artificial cells containing cells or microorganisms, we need to have a truly biocompatible membrane. A number of groups are working to solve this problem with increasing

success (4-6). Another group extends the artificial cells encapsulated cells approach. This is the use of hollow fibre to encapsulate living cells (Aebischer, Lysaght, Galletti etc)(6). This can be inserted into the desired site. On completion of its function, it can be removed. This has advantage in those situations where a large amount of cells are not required, especially in certain central nervous system conditions like Parkinson's disease. However, this is not under the present discussion on artificial cells. References are available elsewhere on this exiting approach(6).

With the present progress in recombinant technologies a number of cell lines and microorganisms with useful properties have become available. For example, we are studying the microencapsulation of microorganisms to remove cholesterol, urea and ammonia (7). This includes the use of genetically engineered E.Coli by Prakash & Chang (7).

ARTIFICIAL CELLS AS A CARRIER FOR ENZYMES, MULTIENZYMES, PEPTIDES, HORMONE AND DRUGS.

A. Enzyme artificial cells in enzyme therapy.

In 1968 we implant catalase artificial cells into mice to replace enzyme defects in an inborn error of metabolism, acatalasemia(8). In 1971 we use asparaginase artificial cells in mice with lymphosarcoma(8). The need to inject this or to use this in extracoporeal applications has delayed its clinical applications. In 1987 we solve this problem by giving orally artificial cells containing phenylalanine ammonia lyase(8). This successfully replaces the enzyme defects in a rat model of phenylketonuria. Our recent finding of an extensive entero-recirculation of amino acids between the body and intestine(1989,1991)(8) explains this effectiveness. This new finding also allows more extensive use of orally administered enzyme artificial cells for the selective removal of other amino acids (8). We have carried out clinical trial of enzyme artificial cells. in 1989. This is the use of orally administered artificial cells containing xanthine oxidase. This effectively lowers hypoxanthine in a patient with hypoxanthinuria (Lesch-Nyhan Disease)(1989)(8). Large scale clinical trial for this, PKU and other conditions has to wait for biotechnology to come out with less costly enzymes.

Most enzymes in biological cells function as complex enzyme systems. Artificial cells can be prepared to contain multienzyme systems with cofactor recycling(9). We use this approach to convert urea and ammonia into essential amino acids like leucine, isoleucine and valine (9). We also prepare artificial cells containing hemoglobin with pseudoperoxidase activity and glucose oxidase to remove bilirubin (9).

B. Biodegradable Artificial Cells as drug carriers.

Another area is the use of biodegradable artificial cells for drug delivery. Biodegradable cross-linked protein membrane artificial cells have been available since 1964 (1). The first artificial cell prepared from synthetic biodegradable polymer, polylactic acid, was reported in 1976 (Chang 1976)(10). We showed that the release rates of insulin could be varied over a wide range. Both of these approaches are now being used in drug delivery for medications, hormones, peptides and proteins by a number of groups(10). In 1969, we prepared lipid membrane artificial cells using a bilayer lipid complexed to ultrathin cross-linked protein membranes(10). Gregoriadis in 1972 used Bangham's 1961 liposomes with onion-skin like lipid structure to microencapsulate enzymes (10). Liposomes are multiple lipid layers onion-skin-like microspheres. Since the content for these are mostly lipid, very small amount of material can be encapsulated. Workers in liposomes therefore turned to preparing small sub-micron artificial cells with a single bilayer lipid membrane. However, they continue to call these liposomes (10). These submicron bilayer lipid membrane artificial cells are now used extensively in drug delivery (10). Magnetic artificial cells prepared by the inclusion of magnetic materials with other biological materials has been reported sometime ago (Chang 1966) (2). This allows them to be localized with external magnetic fields. Kato in Japan more recently shows that magnetic field applied outside the body can direct artificial cells containing magnetic materials and medications to specific sites. More recently magnetic artificial cells are also used in bioreactors. More details on drug carriers will be discussed by Professor Langer in the next article.

CONCLUDING REMARKS:

It should not be too surprising that artificial cells have many medical, biotechnological and other applications. After all, its counterpart, biological cells, are the important basic functional units of all human, animals and living organisms. The following 1972 comment on artificial cell is still relevant(1):

"Artificial cell is a concept. The artificial cells prepared are physical examples for demonstrating this concept. There is no doubt that modification of the present system or completely different systems can be made available to further demonstrate the feasibility of artificial cells..." (Chang 1972)(1)

Indeed since 1972 there are many extensions and modifications. This is clearly shown by the many papers being presented at this congress. With increasing interests in biotechnology and public demands one looks forward to increasing extensions and modifications of the area of artificial cells.

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EDITORIAL

IMMOBILIZATION BIOTECHNOLOGY AND DRUG DELIVERY

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Cambridge, MA 02139-4307, U.S.A.

Approximately half the abstracts submitted for this conference are on immobilization biotechnology and drug delivery. There are several editorial comments that we would like to make in order to introduce this area.

One of the areas that will be discussed in this conference is drug delivery systems. In the last few years, we have witnessed an explosion in research in creating these types of systems. This research is extremely important because it can permit new therapies to exist, and in many cases, decrease the cost that it takes to create a new therapy compared to creating a new molecular entity. Research in this area involves synthesis of new materials, the design of novel biodegradable polymers, the development of new mucoadhesive polymers, and novel methods of microencapsulation. Many of these topics will be discussed at this meeting. One of the major challenges in drug delivery today is being able to develop drug delivery systems for large molecules such as proteins, anti-sense, oligonucleotides and gene therapy agents. By using polymers such as lactic/glycolic acid copolymers, ethylene-vinyl acetate and other polymers, this has been achieved in animal models, and in some cases in humans. Although peptides and proteins were first slowly released from biocompatible polymers in 1976, it was not until 1989 that commercial systems such as Lupron Depot were clinically available to release peptides such as lutenizing hormone releasing hormone analogues. Such systems are now widely used all over the world for the treatment of prostate cancer and endometriosis. In addition, transdermal systems

are being developed to deliver molecules of different sizes. Seven drugs using such systems are already used clinically. Another very exciting area is the development of mucoadhesives for drug delivery. Such adhesives can be used to improve the delivery of drugs orally and also have applications in delivering drugs in other sites of the body. The development of novel microcapsules such as double-walled microcapsules also provides a novel way of improving parenteral delivery. By designing such microcapsules, the delivery systems can be made to provide either constant release or pulsatile release. The latter may be particularly important in new areas of controlled drug delivery such as improved vaccine delivery systems.

Another area that we are emphasizing in this meeting is tissue engineering. Tissue engineering is a relatively new scientific discipline that is having a dramatic effect on science and medical care throughout the world. Tissue engineering is an interdisciplinary field that applies the principles of engineering and life sciences toward the development of biological substitutes that restore, maintain, or improve tissue function. Three general strategies have been adopted for the creation of new tissue: 1) isolated cells or cell substitutes, 2) tissue inducing substitutes such as growth factors, 3) cells placed on or within matrices. Both closed and open systems are being developed for tissue engineering. In closed systems, the cells are isolated from the body by a membrane which allows permeations of nutrients and wastes but prevents large macromolecules such as antibodies or immune cells from destroying the transplant. These systems can either be implanted or used as extracorporeal devices. They can also be designed as microcapsules, or as larger macroencapsulated systems. Open systems have also been used. In this case, cells are attached to matrices which are then implanted, and then become incorporated, into the body. Matrices are fashioned from natural materials such as collagen or synthetic polymers.

A number of exciting areas of tissue engineering will be discussed at this meeting. These include new ways of using hepatocytes to provide liver function, novel approaches for immobilizing ligands to improve cell and tissue interactions, engineering recepter mediated cell behavior, macromolecular implants containing living cells, bioreactors for expanding hematopoetic progenitor cells, large scale *in vitro* cultures for tissues for transplantation, membrane-based artificial organs, clinical applications of tissue engineering, and new methods for using materials science and engineering to produce tissue engineered devices.

Tissue engineering has already reached the point where tissues such as skin are being engineered to treat burn patients. Many of the other tissues under study will undoubtedly be used clinically in the next decade. The talks at this meeting in tissue engineering are intended to introduce this important topic into the area of artificial cells and immobilization biotechnology.

In addition, other important topics will be examined in this conference. New ways of using enzymes, such as heparinase, and novel approaches of creating biosensors will be examined. Biocompatibility and new approaches in biomaterials science will also be discussed.

XI Congress of the International Society for Artificial Cells, Blood Substitutes and Immobilization Biotechnology (ISABI)

July 24-27, 1994

Sheraton Boston Hotel and Towers

Boston, MA, USA

ART. CELLS, BLOOD SUBS., AND IMMOB. BIOTECH, 22(5), xxi (1994)

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ART. CELLS, BLOOD SUBS., AND IMMOB. BIOTECH, 22(5), xxiii (1994)

WELCOME

Dear Colleagues and Friends:

It is a pleasure to welcome you to the **XI Congress of the International Society for Artificial Cells, Blood Substitutes, and Immobilization Biotechnology** which will be held in Boston, Massachusetts from July 24 - 27, 1994.

The new name of the society reflects the merging of international blood substitutes groups with the International Society of Artificial Cells and Immobilization Biotechnology. The International Symposium of Blood Substitutes will continue to meet every other year (odd years). By bringing together these groups and expanding the scope of the Society, this Congress will be broadened to include immobilization biotechnology, blood substitutes, artificial cells, hemoperfusion, sorbents, drug delivery systems, and related topics. This Congress brings together important areas of research development, clinical applications, and industrial production in one major international forum.

A full program with invited lecturers and abstract presentors as well as a schedule of talks are listed in Final Program. Much of the up-to-date scientific exchange will come from submitted abstracts.

We hope that your stay in Boston will be pleasant and scientifically fruitful.

Robert Langer
Congress President

ACKNOWLEDGEMENTS

The XI Congress of the International Society for Artificial Cells, Blood Substitutes and Immobilization Biotechnology (ISABI) has received up to now contributions or commitments for contribution from:

U.S. Army Medical Research and Development Command, Frederick, Md, USA ($5,000)

Hemosol Inc., Etobicoke, Ontario, Canada ($1,000)

Alliance Pharmaceuticals, San Diego, California, USA ($2,000)

Marcel Dekker Publisher, New York, has published and contributed at no cost to the Congress 1000 copies of an issue of the Artificial Cells, Blood Substitutes and Immobilization Biotechnology, An International Journal. This issue contains the abstracts of the XI Congress of the International Society for Artificial Cells, Blood Substitutes and Immobilization Biotechnology.

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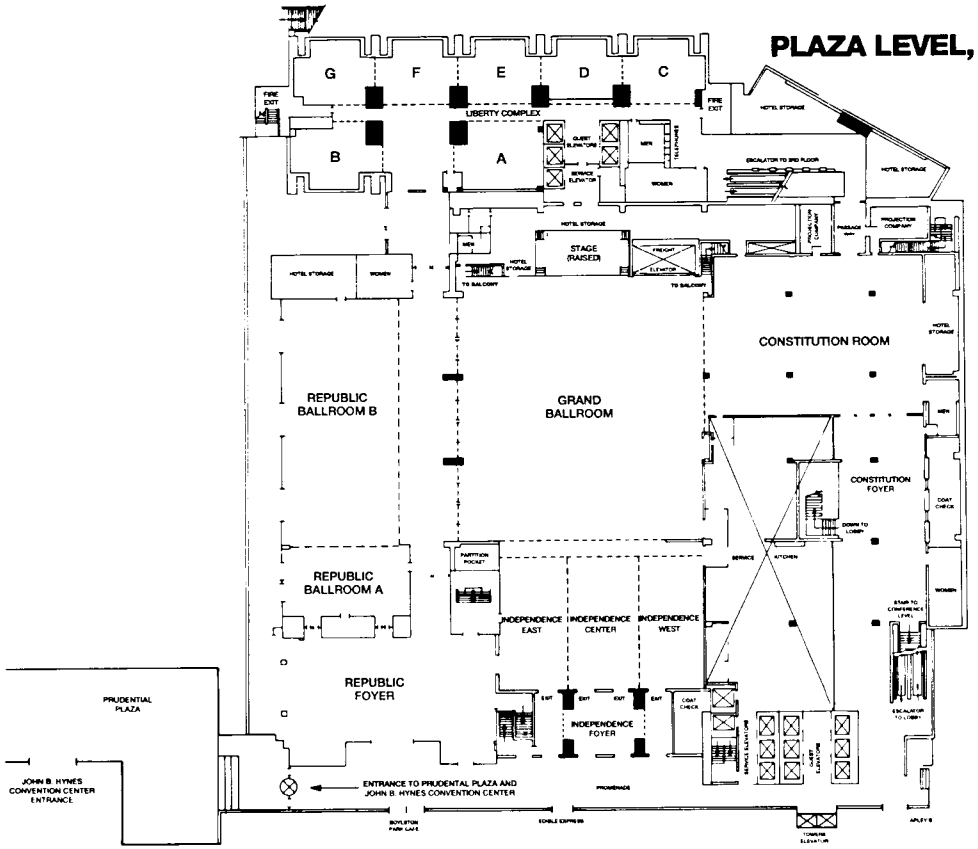

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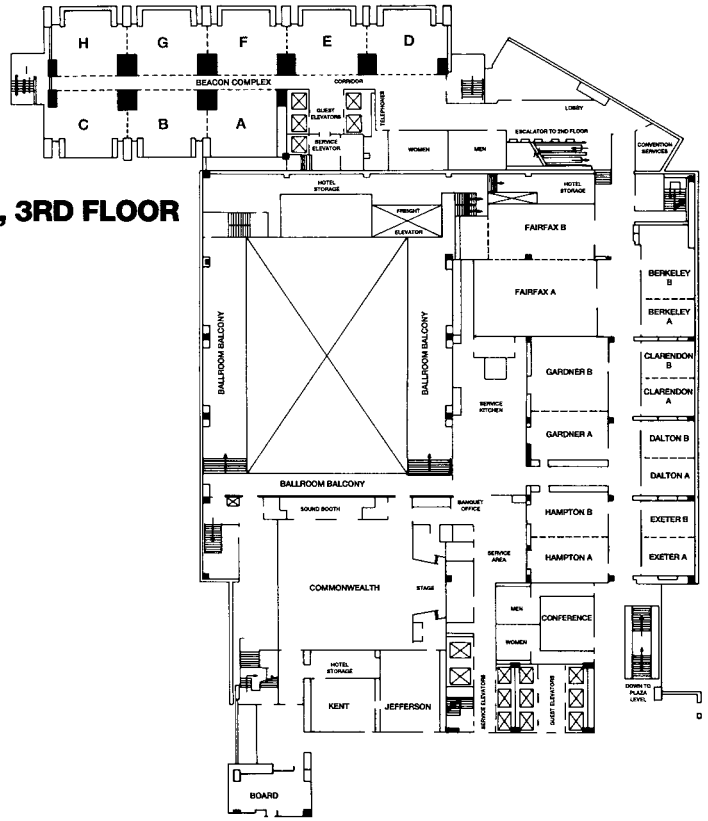

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ABSTRACTS

XI Congress of the International Society for Artificial Cells, Blood Substitutes and Immobilization Biotechnology (ISABI)

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ART. CELLS, BLOOD SUBS., AND IMMOB. BIOTECH, 22(5), A1–A74 (1994)

ABSTRACTS ON

ARTIFICIAL CELLS &

IMMOBILIZATION BIOTECHNOLOGY

A VARIETY OF CLINICAL APPLICABILITIES OF IMMOBILIZED DEXTRAN SULPHATE AS LIPOPROTEIN ADSORBENT AND AVOIDANCE OF ANAPHYLACTOID (ANION-BLOOD CONTACT) REACTION IN ITS USE

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Dyslipidemia including hyper-LDL(low density lipoprotein) cholesterolemia which is very often refractory to dietary /medical treatments is known to be a risk factor of many arteriosclerotic lesions. An extracorporeal procedure of plasma adsorption, LDL adsorption, utilizing dextran sulphate as a ligand immobilized on cellulose gel has been clinically applied in a variety of dyslipidemic conditions as listed below. Its usefulness in secure reduction of the serum LDL level and consequent symptomatic improvements have been confirmed.

Familial hypercholesterolemia (FH) : A regular repetition of the LDL adsorption ameliorates hyper-LDL cholesterolemia as resulting in regression of the multiple stenoses in the coronary arteries.

Focal glomerular sclerosis(FGS) : A session of the LDL adsorption improves kidney function and reduces a urinary protein excretion in FGS patients with dyslipidemia.

Arteriosclerosis obliterans(ASO) : More than 60 ASO patients with dyslipidemia have been treated by the LDL adsorption in our center. In over 80 % of the patients, marked improvement in clinical symptoms such as leg pain/intermittent claudication has been brought out.

Hemodialysis-relevant dyslipidemia(HDDL) : HDDL which develops in the long-term HD patients has been treated.

Transplantation-relevant dyslipidemia(TXDL) : TXDL with deterioration of the transplanted kidney has been treated by the LDL adsorption. Kidney function improves.

Anaphylactoid reaction which is tentatively explained as a result of release of bradykinine in contact of blood with polyanionic material of the adsorbent, dextran sulphate, may develop, in particular, while an angiotensin-converting enzyme inhibitor is administered as a depressant. However, it can be avoided in a use of nafmostat mesilate, a protease inhibitor, as an anticogulant.

PHAGOCYTOSIS OF BIODEGRADABLE POLYMERIC PARTICLES

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Recently we prepared injectable polymeric particles in micron-size range as carriers of radiolabels and drugs for diagnosis and theraphy. Monosize polyethylcyanoacrylate (PECA) microspheres with different sizes (1-4 μm) were prepared by phase inversion polymerization of ECA. Polylactide (PLA) and Polylactide/polyethyleneglycol (PLA/PEG) particles with an average size of 1.5 μm were obtained by solvent evaporation from the respective polymers produced by us. Albumin and fibronectin were also physically adsorbed on these particles. Phagocytosis of these different particles were evaluated by using both fresh human blood, and homogeneous mouse peritoneal and lung macrophage suspensions. Particles were incubated with the respective liquid phases at 37°C for 20 min. Phagocytosis were followed by phase-contrast microscopy. The number of particles phagocytosed by cells decreased by increasing the particle size. PLA based particles phagocytosed more than PECA microspheres. Incorporation of PEG in PLA particles significantly reduced the particle uptake. Albumin coating caused a pronounced change in the phagocytosis. None of the albumin coated particles was phagocytosed by leucocytes. There were very few albumin coated particles uptaken by macrophages. Fibronectin significantly increased phagocytosis both by leucocytes and macrophages.

REPAIR OF DAMAGED CARTILAGE WITH CULTURED CHONDROCYTES

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The purpose of this study is to evaluate the effects of cultured autologous and allogeneic chondrocytes on healing of articular cartilage. Twelve adult canines were employed as the animal model. Chondrocytes retrieved from the non-articulating cartilage of the left femoral chondyle of three dogs were grown and expanded *in vitro* for 3 weeks. After this 3 week period, two chondral defects (4 mm diameter; 1 mm depth) were created in the interchondylar notch of the right knee. Periosteum was retrieved from the proximal tibia and sutured to the cartilage adjacent to the defect and sealed with fibrin glue. Cultured autologous chondrocytes were then implanted under the flap into the defect at a cell density of 2 x 10^6 cells/50 µl culture media/defect. Autologous and allogeneic chondrocytes were genetically tagged with the beta-galactosidase reporter gene via defective retrovirus transduction. Cell-free defects, with or without periosteal flaps, served as controls. Three dogs were employed for each group. Treated knees were immobilized by external fixation for 10 days. Six weeks following surgery, defects were retrieved and analyzed by gross appearance, histology and immunohistochemistry. Cell-free defects with or without periosteal flap consisted of a fibrous layer containing fibroblast-like cells. This fibrous tissue is composed of a matrix containing type I collagen and not type II collagen. Type II collagen was evident in the cartilage adjacent to the defect. Autologous chondrocyte-implanted defects contained both chondrocytic and fibroblastic cell types. Beta-galactosidase labelling indicated that the implanted chondrocytes were primarily along the periphery of the defect and appeared to be fully integrated along the edges with the host adjacent cartilage. The matrix surrounding these cells tested positive for type II collagen and sulfated proteoglycan. Allogeneic cells implanted into the defects did not persist. However, overt immunological rejection by the host was not apparent. Thus, we have demonstrated using an *in vivo* canine model that cultured autologous chondrocytes can function as biomaterials for the repair of focal defects in otherwise normal articular cartilage.

CLINICAL APPLICATIONS OF HEPARINASE

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IBEX Technologies
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We have examined the development of immobilized heparinase reactors for use in extracorporeal therapy to remove heparin following cardiopulmonary bypass and dialysis. Our goals in this project were to achieve the removal of 50-80% of the heparin in human blood at process rates of 100-1000 mls/minute, depending upon the specific application. Heparinase was covalently immobilized to a variety of support materials in order to achieve this. Kinetic analysis suggested that the catalytic ability of the enzyme was not significantly altered after immobilization. Reactors were prepared using heparinase immobilized to cellulosic hollow fibres in dialysis cartridges. These were hemocompatible but due to mass transfer limitations only achieved limited deheparinization in a single pass under normal operating conditions. Mass transfer limitations could be overcome by inducing flux so that substrate was forced through the fibre wall where the heparinase was immobilized. High flux plasmapheresis hollow fibres removed heparin very efficiently, however plasmapheresis itself is undesirable. Reactors using immobilized beads were also studied. Fluidized beds could not be used in a viscous fluid like blood because at any practical flow rate all the beads would migrate to the top of the reactor. Packed bed reactors could remove virtually all the heparin in a single pass in buffer but in blood there was an increasing resistance to flow. The most promising format is a compromise between the above two cases, a "rigid fluidized bed"; a reactor having heparinase immobilized cellulosic beads dispersed in an inert porous sponge. This overcame mass transfer limitations (50% single pass conversion), did not foul and was found to be the most efficient use of a given amount of enzyme.

CONJUGATION OF BILIRUBIN INTO MONOCONJUGATE (BMG) AND DICONJUGATE (BDG) BY EACH OF ENCAPSULATED HEPATOCYTES, FREE HEPATOCYTES OR HEPATOCYTE HOMOGENATE

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The present study shows that homogenized rat hepatocytes, whole hepatocytes and hepatocytes encapsulated in an alginate-polylysine-alginate (APA) artificial membrane when incubated with bilirubin and UDP-glucuronic acid, can perform an important liver function, *i.e.* bilirubin conjugation. The enzymatic activity of the homogenized hepatocytes is higher than that of whole hepatocytes or that of encapsulated hepatocytes, because for the homogenized cells the enzyme and the substrate are free in solution and thus there is no mass transfer resistance to their interaction. However, notwithstanding the presence of 2 membranes (the APA) artificial membrane and the natural cellular membrane) the encapsulated hepatocytes produced conjugated bilirubin. Bilirubin monoconjugate (BMG) and bilirubin diconjugate (BDG) were both produced, however there was significantly more BMG than BDG formed. The kinetic data of the enzyme UDP-glucuronyltransferase (UDP-GT) suggest that it is a multisubunit enzyme in which there is cooperative binding of substrate to the subunits. The kinetic data shows that the binding of bilirubin occurred with positive cooperativity. The cooperativity of UDP-glucuronic acid binding was mixed since it was negative at low concentrations and positive at high concentrations.

ALGINATE-POLYLYSINE-ALGINATE MICROCAPSULES AS A BIOARTIFICIAL ORGAN

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**Dept Physiology, Univer Toronto, Ontario, Canada

In the bioartificial organ, pancreatic islets, parathyroid cells or dopamine-secreting PC-12 cells could be encapsulated in alginate-polylysine-alginate three layer microcapsules(MC), whose diameter is about 0.5 mm. Spherical and smooth MC could be formed utilizing a purer sodium alginate and by keeping the 1.5% alginate gel about 30 cps. It was observed insulin and lactalbumin (MW.14200) easily diffused into and out of the MC, however the membrane itself is not permeable for hemoglobin and albumin(MW. 66,000). In the present study we have been successful in encapsulating rat's and canine's pancreatic islets and transplanting them into diabetic mice and rats, thereby restoring normoglycemia for three months without the use of immunosuppressive agents. Human parathyroid cells were isolated from surgically resected parathyroid adenomas, encapsulated and implanted in rats on which total parathyroidectomies had been performed. One week after implantation, the serum calcium level increased from 1.15 mM/L in parathyroid status to 1.28 mM/L and up to 1.82 mM/L after four months of implantation. The intact parathyroid hormone concentration increased from undectable level to the range of 6.5 and 8.5 pg/ml in the 10th week of implantation. Encapsulated PC12 cells, a catecholaminergic cell line derived from a rat pheochromocytoma, exhibited good survival, proliferated and spontaneously released dopamine for at least two months. These results suggest that a great clinical potential to be developed for bioartificial organs.

STUDY OF MICROENCAPSULATION FOR PITUITARY TRANSPLANTATION:CAPSULE PREPARATION AND IN VITRO STUDY

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Microencapsulation has been widely studied in many living cell transplantation. We have developed microencapsules using sodium alginate (SA) and Poly-L-Lysin (PLL). A factorial design method of screening was chosen to study the influence of the different experimental parameters on capsules' size and stability. We found that, air flow affects on initial capsules size significantly ($P<0.01$), while PLL molecular weight (MW) and incubation times being directly proportional to the capsules' enlargement when the capsules placed in sodium citrate buffer (SC) in order to liquify the calcium alginate gel core ($P<0.01$). When the capsules were continuously shaken in an imitative in vivo environment (37°C, 250 RPM), those capsules made by optimal parameters combinations were still intact till 28 days and 50% broken within 60 days, while others more than 50% broken within merely 2 hours. The permeability of the microencapsule membrane (CM) was tested, the results revealed that it is permeable to pituitary hormones, TSH, GH, PRL, and impermeable to immunoglobulins when producing CM with PLL MW of 80,000. (Chen & Bao, 1994). We also encapsulated human pituitary adenoma cells(obtained from transsphenoidal surgery) and cultured it with RPMI 1640 for one week, found that the adenoma cells both encapsulated or unencapsulated could secrete hormones ($P>0.05$). Our preliminary study suggested that microencapsules made of PLL and SA offer suitable semipermeable characteristics and could be used for pituitary transplantation study in vivo.

REF: Chen & Bao, Chinese Medical Journal. 1:in press. 1994

POLYDISPPERSE DEXTRAN AS MODEL DIFFUSANT TO STUDY THE EFFECT OF ENCAPSULATION PROCEDURE AND PROCESS PARAMETERS ON PERMEABILITY OF ALGINATE-POLYLYSINE-ALGINATE (APA) MICROCAPSULES FOR BIOHYBRID ORGANS

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Control of membrane permeability of APA microcapsules is of particular importance in the design of biohybrid organs. The microcapsule membrane must allow the diffusion of metabolites including peptides and small proteins and the exclusion of leucocytes and immunoglobulins (MW > 150 000). It is difficult to accurately measure simultaneously the diffusion of biomolecules covering such a large spectrum of molecular weights. Furthermore, model diffusants differ in charge, surface properties and molecular structure. A new method that measures the concentrations of dextran fractions covering a span of MWs (Coromili & Chang, 1993) is used to study membrane permeability. By HPLC, changes in concentrations of all the molecular weight fractions of dextran can be carried out simultaneously in one experiment. Ongoing studies show that the microcapsule membrane does not show a distinct MW-cut-off to dextran but rather spans a wide MW range suggesting a non-uniform membrane. Differences in permeability of three types of APA microcapsules designed for biohybrid organs are compared: (i) standard single membrane, (ii) single membrane prepared with a novel procedure to eliminate the entrapment of cells within the membrane matrix and (iii) double membrane where intracapsular alginate is minimized for improved intracapsular diffusion and cell contact. By varying encapsulation process parameters and procedure, this method of characterizing membrane permeability can be effectively used to select for microcapsules with the most narrow and distinct molecular weight cut-off as close to 150 000 as possible.

REF: Coromili & Chang, J.Biomat.Art.Cells,Artif.Organs,21(3), 427-444, 1993

DEGRADATION AND STABILIZATION OF IMPLANTABLE POLYMERS

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Polymeric biomaterials intended to perform in "permanent" or biodegradable medical devices have been shown to degrade chemically *in vivo* by two major mechanisms: hydrolysis and oxidation(1). Materials designed to biodegrade generally break down by hydrolysis. Mechanisms of simple hydrolysis, acid, base, and enzyme catalysis have been documented. Catalytic hydrolysis of hydrophobic polymers is generally diffusion-controlled and short-range, occurring from the surface inward. Subsequent cracking exposes more surface area for attack. Catalysis of hydrogels depends on their permeability to the catalysts, with basic and acidic ions being quite effective throughout the bulk because of their small size. Autocatalysis may occur if acidic or basic species are generated during degradation. Insights into oxidative degradation of polymeric biomaterials generally have come from studies of polymers intended for long-term implantation(1,2). The predominant mode of oxidative attack by the host involves phagocyte-derived oxidants such as superoxide (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH\bullet$), hypochlorite (OCl^-) and nitrogen species: peroxynitrite ($ONOO^-$) and nitric oxide (NO)(3). Degradative changes may be enhanced under conditions of mechanical stress to the biomaterial. As with hydrolysis, this type of oxidation is diffusion-controlled and short-range. Occasionally, medical devices contain components which can interact with polymers to effect oxidative changes(1). Corrosion products from conductor coils of cardiac pacemaker leads, for example, have induced oxidative degradation of polyurethane insulation sheaths. Degradation of hydrolysis-susceptible polymers may be retarded by maintaining neutral pH in their vicinity and by protecting them from enzymatic catalysis. Oxidation effects may be minimized by: prevention of adhesion and activation of phagocytes; use of antioxidants; minimization of mechanical stresses; prevention of metal corrosion in the presence of the polymer; and use of oxidation-resistant polymers.

(1) Coury, AJ and Stokes, KB, "Chemical and Biochemical Degradation of Polymers," *Biomaterials Science: An Introduction*, Ratner, BD et al (Eds.), Academic Press, in press

(2) Rimnac, CM et al, *J. Appl. Biomater.*, 5:17-21. Spring 1994

(3) Sutherland, K, et al, *J. Clin. Invest.*, 92:2360-2367. Nov. 1993

IMMUNOADSORPTION OF ANTI-DNA ANTIBODIES BY USING DNA IMMOBILIZED PHEMA MICROSPHERES

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Polyhydroxyethylmethacrylate (PHEMA) based sorbents carrying DNA were investigated for spesific sorbent hemoperfusion for removal of anti-DNA antibodies. PHEMA microspheres (about 200 μm) were prepared by suspension polymerization. The hydroxyl groups on the PHEMA microspheres were activated by CNBr in an alkaline medium (pH: 11.5) and the ligand, DNA was then immobilized. The optimal DNA immobilization conditions were as follows: the CNBr initial concentration: 10 mg/ml; pH of the immobilization medium: 5.0; and the initial concentration of DNA: 2 mg/ml. The maximum amount of immobilization, which was achieved at the optimal conditions, was 2.75 mg DNA/g PHEMA. The plasma samples obtained from a patient with Systemic Lupus Erythematosus (SLE) were treated with the PHEMA/DNA sorbents carrying different amounts of DNA (0.70-2.75 mg DNA/g PHEMA), in batch reactors. The non-specific adsorption of anti-DNA antibodies on the plain PHEMA microspheres was low (about 0.19 mg anti-DNA antibody/g PHEMA). While higher adsorption values (up to 40 mg anti-DNA antibody/g PHEMA) were observed in the case in which DNA carrying PHEMA microspheres were used, due to specific interaction between immobilized DNA and anti-DNA antibody molecules.

POLYMERIC MICROCARRIERS FROM PDLLA HOMOPOLYMERS AND PDLLA/PEG COPOLYMERS AS DRUG CARRIERS

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Poly(DL-lactide)s (PDLLA; MW:10000-100000) were prepared by ring opening polymerization of DL-lactide, and then were transesterified with polyethylene glycol (PEG; MW: 4000-5000). Polymeric particles with different sizes (1-10 μm) both from PDLLA homopolymers and PDLLA/PEG copolymers were produced by solvent evaporation by using different amounts and types of solvents (solvents: methylene chloride and chloroform) and by stirring the dispersion medium both with a mechanical strirer (500-2000 rpm) and by sonication (250 W). The average size and size distribution of particles were determined by SEM. Size of the particles reduced by increasing the rate and power of stirring. The size distribution of microspheres became narrower with increasing PDLLA molecular weight. A model drug, rifampicin, a tuberculostatic agent was loaded within these particles. Different levels of drug loading (5-97 mg drug/g polymer) were achieved by changing the amount and type of solvent, and initial drug concentration. The highest loading (97 mg drug/g polymer) was achieved at the following conditions: solvent type: methylene chloride; amount of solvent: 100 ml/g polymer; initial drug concentration: 500 mg drug/g polymer. Release from these matrices was studied in vitro. The release medium conditions were as follows: medium: phosphate buffer; pH: 3-7.4 and 9.8; and temperature: 4°,37° and 45°C. Release rate increased by increasing pH and temperature. Higher rifampicin release rates were obtained with the particles prepared from low molecular weight PDLLA, which degraded faster.

ARTIFICIAL-CELL-IMMOBILIZED HEPATOCYTES AS A BIOARTIFICIAL LIVER

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The prospect of treating severe and chronic liver diseases with isolated hepatocytes is fast becoming a realistic goal. Advances in tissue culture and cell immobilization techniques significantly enhance the therapeutic potential of isolated hepatocytes for treating a wide array of liver disorders. Artificial-cell-immobilized hepatocytes (i.e., microencapsulated hepatocytes) is one such advance that has demonstrated efficacy in providing liver-specific function in various experimental animal models of liver disease. In essence, microencapsulation facilitates the immunoisolation of transplanted cells in a foreign environment. Thus, it provides a unique and innovative technique for cell transplantation without the need for immunosuppression. We showed that, unlike free hepatocytes which evoke a strong immunogenic response following xenotransplantation, microencapsulated hepatocytes are rendered non-antigenic even after repeated exposure to the host's immune system. In short-term studies (i.e., 7-10 days) we showed that intraperitoneally transplanted microencapsulated hepatocytes provided sufficient metabolic support to significantly improve the survival of animals with fulminant hepatic failure. In long-term transplantation studies (>1 month) we demonstrated the effectiveness of microencapsulated hepatocytes in reducing congenital hyperbilirubinemia in Gunn rats for periods of 4-6 weeks. Beyond this period, transplanted microencapsulated hepatocytes underwent considerable degeneration. Recently we showed that cell-surface interactions play an important role in the onset of these degenerative changes. They were delayed if liver basement-membrane proteins were used to prepare the microcapsules. Sustained correction of hyperbilirubinemia for at least 6 months was also demonstrated in the Gunn rat by repeated monthly transplantations of microencapsulated hepatocytes. In other studies we showed that microencapsulated hepatocytes can be successfully cryopreserved for several weeks with little or no loss in viability and function. This observation is important in developing a liver support system that can be easily stored and be available on demand. Our recent efforts have focused on the use of microencapsulated fetal porcine hepatocytes in liver support systems. Porcine hepatocytes are advantageous because they are hardy and can be readily obtained in large quantities. Our preliminary results suggest that these cells are ideal for use in humans where large volumes of hepatocytes would be required to treat severe liver disease.

NEW FAMILY OF HYDROGEL AS MATRIX FOR ENZYME IMMOBILIZATION BIOMEDICAL APPLICATIONS

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The development of hydrogels in the biomedical field is still increasing. The high water content of hydrogels makes them good candidates for biomedical applications. Recently, new hydrogels based on polyethylene glycol crosslinked with albumin have been described (1, 2). The equilibrium water content (EWC) of these hydrogels reached high values (96-98 % in water). The presence of PEG lowers the immunogenicity as it was largely demonstrated with protein or enzyme modification by PEG and reduces protein adsorbtion and cell adhesion wich are good prerequisites for biocompatibility. Among the many possible applications of these hydrogels, they can be usefull as matrix for enzyme immobilization. Acid phosphatase (AP) was choosen as a model to study the effect of PEG molecular mass on the diffusional restrictions of substrate and product when the enzyme is covalently immobilized into hydrogel microbeads (210-700 μm) used in a flow system. Asparaginase (Asnase) hydrogels were also prepared and implanted into rats in pastille form. This enzyme has been implicated in the treatment of leukemia in children. Biocompatibility study based on histochemistry and immune response to the implant will be discussed. The bioreactor effectiveness (corresponding to plasmatic asparagine depletion) is evaluated. In both immobilized systems (i.e. with AP and Asnase hydrogels), the Kms for pnPP and asparagine, respectively, do not strongly increased after immobilization. This suggests low diffusional restrictions through the polymeric device. Moreover, the operational stability of both immobilized enzymes at 37 °C was markedly improved compared with soluble enzymes.

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NOVEL POLYMERIC MICROCARRIERS FOR BIOMEDICAL APPLICATIONS: MICROPOROUS HYDROGELS CARRYING DIFFERENT FUNCTIONAL GROUPS

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Microporous hydrogels in spherical form in the size range of 50-250 μm were produced by a novel copolymerization of ethyleneglycoldimethacrylate with different comonomers, namely acrylic acid, 2-hydroxyethylmethacrylate and acrylamide. Therefore, swellable polymeric microspheres having different functional groups, i.e., carboxyl, hydroxyl and amine groups were produced. Benzoyl peroxide and poly(vinyl alcohol) were utilized as the initiator and stabilizer in the copolymerizations, respectively. Toluene was included in the copolymerization recipe to achieve the microporous hydrogel structure. The existence of functional groups in the bulk and surface structure of polymeric microspheres was confirmed by FTIR and FTIR-DRS measurements. Potentiometric titration was applied for the determination of carboxyl content of the copolymer microspheres. The bulk and surface morphology of the copolymer microspheres were examined by scanning electron microscopy. The results indicated that, the functional monomer distributed homogenously in the resultant porous polymeric structure. The swelling charecteristics of the microspheres were also studied in an aqueous buffer medium. The diffusion of water into the dry cross linked microspheres could be observed by color change of the microspheres under optical microscope. The swelling ratios up to 150 % were obtained with the carboxylate modified microspheres.

STUDY OF THE IMMUNOLOGICAL RESPONSE TO TISSUE ENGINEERED CARTILAGE IN VIVO

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Techniques of tissue engineering using bioerodable polymers and injectable gels onto which transplanted cells are seeded are promising ways to provide new tissue of similar quality to the original in specific areas for repair or augmentation. It is not known, however, how tissue engineered constructs will perform across allogeneic combinations and whether they will be rejected or not. It is also not known which delivery method affords the best immnunological protection for the transplanted cells. The behavior of cartilage constructs created across a major histocompatibility barrier using two different cell delivery schemes is being investigated. ACI rats are used as source of chondrocytes and as syngeneic recipients. Lewis rats are utilized as allogeneic recipients with a major histocompatibility barrier to the ACI strain. Athymic mice are used as immunotolerant recipients for control purposes. Cartilage is harvested from the xyphoid of ACI rats under sterile conditions and dissociated into chondrocytes by incubation in a collagenase solution. The retrieved chondrocytes are then implanted onto the recipient animals using two different delivery systems. The first method utilizes the delivery of a calcium alginate-chondrocyte mixture via injection under the pannus cunniculus on the dorsum of the recipient animals. The second method utilizes the surgical implantation on the dorsum of the recipient animals of a fibrous polyglycolic acid (PGA) polymer that had been seeded with chondrocytes for one week in culture medium. Animals from each of the three groups (syngeneic, allogeneic, athymic) receiving the implants are sacrificied at 2, 4, and 8 weeks post-implantation. The structures retrieved from the sites of the implants are analyzed histologically with hematoxylin-eosin, Alcian blue, and Masson's trichrome, and immunohistochemically with monoclonal antibodies specific to rat CD4 T cell, CD8 T cell, B cell, and macrophage. This analysis permits the identification of neocartilage constructs and the evaluation and quantification of the extent of the local immune response to these constructs at the different times post-implantation. This study attempts to determine the nature and evolution in time of the immunological processes that exist when neocartilage constructs are created in syngeneic and allogeneic combinations and in an immunologically tolerant group. It also enables an evaluation of two cell transplantation delivery systems (calcium-alginate solution and polyglycolic acid polymer) in terms of their immunological protection to tissue engineered constructs in the host. The ability to create minimally-immunogenic constructs in allogeneic combinations would suggest the possibility of utilizing cultured chondrocytes from different donors for tissue transplantation.

DRUG RELEASE FROM NEW BIOARTIFICIAL HYDROGEL

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Controlled releasing (CR) systems of drugs have been widely studied to evaluate their advantages in delivering biologically active substances. They maintain the drug concentration below the toxicity level allowing elimination of indesirable side effects and repeated dosing [1]. Among the numerous systems leading to CR devices, hydrogels have recently attracted significant attention because of their high water content and their relatively good biocompatibility [2,3]. In this paper, we present the use of a newly synthetized hydrogel [4] as biomaterial for CR system, and its evaluation in releasing of theophylline and some other substances.

Our hydrogel is an artificial biopolymer obtained by copolymerisation of functionnalized poly(ethylene glycol) (PEG) with bovine serum albumin (BSA). This BSA-PEG hydrogel has a high water content (EWC>96%), good mechanical properties and good biocompatibility. It is shown that release of theophylline, acetaminophen, hydrocortisone and even protein (lysozyme, MW≈17000 Da) from such hydrogel occurs by a diffusion controlled mechanism, fitting a square-root of time relationship. Release process of these drugs from BSA-PEG hydrogel were characterized, and diffusion coefficients and half-life time values of release were calculated for all drugs. The effect of the hydrogel composition was also evaluated. The diffusion coefficients for the release of theophylline from hydrogels obtained from various molecular masses of PEG were determined. An increase in diffusion coefficients is observed when the molecular mass of the PEG is increased, suggesting a wider porosity of the hydrogel. This result allows the release rate to be tailored by varying the composition of the hydrogel.

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ROLE OF BLOOD SUBSTITUTES IN HOLLOW FIBER CELL CULTURE

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Blood substitutes have recently found favor in spinner flask and stirred tank cell culture [1][2]. Specifically, bovine derived hemoglobin has been chemically stabilized and used to enhance oxygen supply to cells in culture. In particular insect cell culture is targeted because these cells are oxygen intensive [2]. Unfortunately published data is limited at this time. However, early results suggest that both cell mass and metabolite production increases when modified bovine hemoglobin is added to cell culture media. We have investigated blood substitutes of various types in hollow fiber bioreactors with different cell types. Some remarkable differences to that in conventional cell culture systems have been discovered. The role of blood substitutes in hollow fiber cell culture is discussed. The data presented have far reaching implications for both biopharmaceutical production and the exvivo expansion of therapeutic cells.

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IMMOBILIZED LIGANDS FOR CELL AND TISSUE INTERACTIONS

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The interaction of adhesion receptors with their protein ligands, such as fibronectin, can be localized to small domains, called the cell-binding domain, on the surface of the protein. The receptor-binding activity of the cell-binding domain can be mimicked by short oligopeptides of the same or related sequence as that of the cell-binding domain, typically 3 to 6 amino acid residues in length. These oligopeptides have been incorporated into the surfaces of polymeric biomaterials, resulting in surfaces for which the cells bear receptors directly, i.e. for which adsorbed adhesion proteins are not necessary. This enables several possible advantages over approaches to promoting cell adhesion with adsorbed proteins, including more effective utilization of the immobilized ligand and the possibility to selectively promote the adhesion of one type of cell. For example, investigation with fibroblast spreading *in vitro* revealed that approximately 10^5 ligands per cell were required to elicit spreading, focal contact formation, and f-actin cytoskeleton organization; this is approximately two orders of magnitude less than that required with the adsorbed protein fibronectin, presumably due to more competent presentation of the small synthetic adhesion ligand compared to the intact protein (Massia & Hubbell, 1991). As an example regarding cell-type selectivity, endothelial cells bear a receptor for the fibronectin sequence REDV (Massia & Hubbell, 1992), while blood platelets do not, and this fact has been used to create biomaterials that may support endothelialization without inducing thrombosis (Hubbell et al., 1991). An important aspect in selectivity is the ability to make derivatizable materials that support very little protein adsorption and nonspecific cell adhesion, and hydrophilic multifunctional polymers have been used toward this end (Drumheller et al., 1994).

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LONG TERM STORAGE OF MICROENCAPSULATED PORCINE ISLETS OF LANGERHANS FOR THE TREATMENT OF TYPE I DIABETES

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Porcine islets of Langerhans, immunoisolated in alginate-polylysine-alginate (APA) microcapsules are being studied as an intraperitoneally injectable bioartificial endocrine pancreas for the treatment of insulin dependent diabetes mellitus (IDDM). In order to create an islet bank, microencapsulated porcine islets were cryopreserved and assessed in a transplantation study. Islets isolated from pigs by collagenase digestion were encapsulated in APA using an electrostatic droplet generator. After 2-3 hours in culture medium, the islets were equilibrated in 2 M dimethyl sulfoxide (DMSO) by stepwise addition, cooled at approximately 1°C/minute to -70°C and then immersed in liquid nitrogen. After thawing at 100°C/minute in a 37°C water bath, the DMSO was removed by dilution in 7.5 M sucrose and the islets cultured in media for 2-3 hours. Viability was tested by static *in vitro* challenge with low (50 mg%), high (300 mg%) or high glucose (300 mg%)+IBMX (0.1 mM) media. The media was assayed for insulin content at 24 hours by RIA. Preliminary results show an insulin response very similar to the unfrozen controls. The insulin secretion of frozen islets in low glucose was 15.6 ± 1.4 μIU/mL/24 hr/islet and increased to 28.1 ± 9.4 μIU/mL/24 h/islet in high and 30.3 ± 3.2 μIU/mL/24h/islet in high+IBMX. The respective secretion for fresh islets from the same organ were 23.2 ± 1.8 μIU/mL/24 h/islet (low), 31.2 ± 8.4 μ IU/mL/24 h/islet (high) and 35.0 ± 3.3 μIU/mL/24 h/islet (high+IBMX). Whereas free islets are easily fragmented and lost during this process, the encapsulated islets and membrane remain intact. The capsule may serve to protect the fragile islets from freezing damage resulting in a retrieval rate very close to 100%. In on-going preliminary studies, transplantation of banked microencapsulated rat and porcine islets into streptozotocin induced diabetic BALB/c mice has resulted in restoration of normoglycemia. This will be a useful adjunct to clinical trials.

TRANSPLANTATION OF CARTILAGENOUS TISSUE GENERATED IN VITRO INTO ARTICULAR SURFACE DEFECTS

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We have optimized a cell culture system in which isolated chondrocytes reconstitute their extracellular matrix similarly to articular cartilage. The purpose of this study was to determine whether the cartilagenous tissue generated in vitro could be used as transplants to resurface damaged joints. Chondrocytes were obtained from rabbit articular cartilage by sequential enzyme digestion (1) and plated as a monolayer at a cell density of $1.5 \times 10^6/cm^2$ on Millicell CM^R filters precoated with type II collagen. The cells were maintained in culture up to three months prior to transplantation. To create articular surface defects, NZW rabbits (3.5 kg) were anaesthetized and a 3mm punch defect was made in the articular cartilage of the patellar groove of the left femur. The cartilagenous tissue, which had formed in culture, was harvested and placed in the defect. An adhesive agent, either Cell-Tak (Collaborative Biomedical Products), or Nexaband Avian (Tri-Point Medical L.P.) was applied topically to the defect immediately prior to transplantation. In selected rabbits, the articular defect was extended into the superficial subchondral bone using a rotary drill. In these animals, the tissue was transplanted in the absence of any fixation. After one week, the rabbits were euthanized and the joints removed. The femur was fixed in 10% formalin, decalcified and paraffin embedded. 5um sections were cut, stained and examined by light microscopy. No tissue remained in those animals in which the transplant was immobilized with Cell-Tak. Transplants fixed into the defect with Nexaband consisted of a condensed thin layer of acellular tissue. Viable cartilagenous tissue was present in the animals in which the transplant had been placed, in the absence of adhesive, into the subchondral bone defects. In conclusion, cartilagenous tissue generated in vitro can survive transplantation but an appropriate method for graft fixation is required.

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INVESTIGATION OF THIOUREA ACTIVATED POLYGLUTARALDEHYDE WITH BOUND Ag(I) OR Pt(II) AS AN ALTERNATIVE TO AVIDIN FOR IMMOBILIZING BIOTIN CONJUGATES

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Avidin, and streptavidin have been used for immobilizing biotin conjugated biopolymers for affinity chromatography, ELISA, biosensors, and DNA hybridization. Avidin-biotin technology provides a means for immobilizing biopolymers very strongly while maintaining bioactivity. However, the biotin-avidin interaction is difficult to reverse resulting in loss of avidin/streptavidin and more importantly, the biopolymer of interest. Although various alternative approaches such as using a cleavable linker attached to biotin and modified avidin/streptavidin have been proposed, these approaches have not seen widespread adoption due to weak affinity, need for extremes in pH for reversing the complex, and high sensitivity to steric effects. We have developed a method for immobilizing Ag(I) and Pt(II) onto a gel filtration media using polymerized glutaraldehyde activated with thiourea. The resultant gel resin has a high capacity (up to 0.1 mmol/ml) and has been shown to be stable and useful even in the presence of relatively high chloride (up to 1 M) and phosphate concentrations (0.25 M). In research with amino acid and biotin chromatography, we have shown that Ag(I) and Pt(II) have a high affinity for thioether groups [Garcia, Kim, and Miles, 1993]. To test the selectivity for binding biotinylated proteins, separate solutions of Biotinylated BSA (b-BSA) and BSA were applied to the gel in the Ag(I) and Pt(II) form. Using the Ag(I) form of the gel at pH=7 (0.05 M phosphate) B-BSA binds and 30% can be eluted using 0.15 M NaCl while no BSA binds to the column. Although BSA contains 35 cysteine and 4 methionine residues, unconjugated BSA does not bind to Ag(I) presumably because the thiol group of cysteine is reduced forming disulfide bonds and the methionine residues are not as accessible as the thioether group of biotin. Unlike the Ag(I) column, a small amount of BSA binds to Pt(II). At pH=4.8 and 1 M NaCl, less than 10% of the applied BSA binds while 50% of B-BSA binds to the Pt(II) column. Currently, we are synthesizing higher molecular weight polyglutaraldehyde which should result in a linking molecule with a significantly higher density of biotin binding sites than avidin. Moreover, the density of sites can be controlled by varying the amount of thiourea covalently linked to glutaraldehyde or by varying the size of the polymer.

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HEPARINIZED SURFACES:

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For the past several years, we have investigated the application of heparin modified surfaces toward the ultimate goal of producing nonthrombogenic surfaces. The approaches used to achieve these goals include the design of heparin releasing polymers, heparin immobilized onto polymer surfaces through polyethylene oxide (PEO) spacer groups, and heparin-PEO-grafted or heparin-PEO-block copolymer coating surfaces. Physical and chemical characterization of the heparinized surfaces established that the bioactivity of heparin was retained during the immobilization processes. Furthermore, all heparinized surfaces demonstrated significant reduction of thrombus formation when in contact with blood. An important parameter in these studies was the effect of PEO as the spacer group. Heparin immobilized surfaces using PEO spacers (graft copolymers) resulted in minimal fibrin net formation and minimal platelet aggregation on the blood contacting surfaces during in vitro, ex vivo and in vivo experiments; in contrast to the rather poor results of heparin directly immobilized on the surface. Triblock copolymers were also synthesized consisting of a hydrophobic block (polydimethylsiloxane), a hydrophilic segment (PEO) and heparin. The graft copolymers and the triblock copolymers were evaluated as vascular graft coatings in vivo. The coated surfaces successfully reduced thrombus formation, minimized protein adsorption, and prevented vascular occlusion after several months implantation in a dog. Several state of the art designs of blood compatible surfaces will be discussed.

HIGH YIELD ISOLATION OF PORCINE HEPATOCYTES FROM SLAUGHTER HOUSE ORGANS

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Most recent strategies for the development of hybrid artificial liver devices are based on the use of hepatocytes. However, for clinical application of such devices, sufficient cell supply (approximately 50 billion hepatocytes per set up) is mandatory. We have developed a modified collagenase perfusion technique in order to harvest high yield of porcine liver cells from slaughterhouse organs. Perfusion and enzymatic digestion of the left medial liver lobe (n=52) resulted in 6.7 $\pm$ 0.3 x 10^6 hepatocytes per gram tissue and an overall yield of 1.5 $\pm$ 0.5 x 10^9 cells per isolation (viability: 93 $\pm$ 2%).

In order to maintain morphological integrity and functional activity of hepatocyte cultures over long-term periods, we have introduced an easy to apply collagen gel immobilisation technique. Integrity of morphology, as assessed by light and electron microscopic follow up, was maintained for 14 days. Stable DNA-contents and low values for alanine-amino-transferase release were measured after a short period of early culture adaptation. Albumin secretion, stable bile acid synthesis and persistent activity of cytochrome P450 IA1 dependant deethylation of 7-ethoxycoumarin indicated functional stability over long-term periods. Urea production could be induced 2 to 4 fold by 10 mM ammoniumchloride for at least 10 days.

Hepatocytes isolated from slaughtered piglet represent an unlimited resource of viable cell material for functional units of bioartificial liver support devices.

Biosensors Based on Intelligent Polymeric Systems

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Polymeric systems that response to specific molecules serve as the basis for technological applications such as biosensors, drug delivery devices and actuators [1]. Two approaches for development of biosensors based on such intelligent polymers will be presented. Incorporation of ionic groups into hydrogel causes its swelling properties to vary with pH. By entrapment of glucose oxidase into the pH sensitive hydrogel, it becomes capable of responding specifically to glucose. Glucose oxidase, causes glucose to be converted to gluconic acid. The gluconic acid, in turn, decreases the pH in the microenvironment of the hydrogel, therefore affects its swelling. The proposed biosensor is based on the change of the hydrogel electrical properties as a result to its swelling when exposed to glucose. The electrical properties of the hydrogel with and without immobilized enzyme in constant voltage or frequency were characterized. Relation between the extent of swelling of the hydrogel at different glucose concentrations and its electrical conductivity was determined. The other approach for biosensors based on intelligent polymers consists of a starch matrix and its hydrolytic enzyme α-amylase, in its non active form. As the activity of the unstabilized α-amylase is calcium dependent, the degradation of starch by α-amylase depends on the concentration of calcium in the surrounding environment. In addition to possible signal detection, the proposed systems have the ability to perform specific function in response to external signals, such as: sensitive separation processes, valve controllers, and responsive drug delivery systems.

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ISLET TRANSPLANTATION USING IMMUNOISOLATION

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Immunoisolation systems have been conceived in which transplanted islets are separated from the immune system of the diabetic host by an artificial barrier. These systems offer a solution to the problem of human islet procurement by permitting use of islets isolated from animal pancreas. Three major types of immunoisolation devices have been studied in our laboratory. These include devices anastomosed to the vascular system as AV shunts, diffusion chambers, and microreactors. In the first type of system, canine and porcine islets were distributed in a chamber surrounding a permselective acrylic membrane (nominal M_r exclusion of 80kD), and the devices implanted into diabetic, totally pancreatectomized dogs. Fourteen of the recipients (11 canine, 3 porcine) maintained graft function (>10 Units/day) for periods of time ranging from two months to more than a year. In the second type of system, the islets were sealed within the acrylic membranes and the chambers implanted into the peritoneum of diabetic, pancreatectomized dogs (canine islets), STZ-induced diabetic rats (canine, bovine and porcine islets), and spontaneously diabetic BB/Wor rats (canine islets). All but one of the dogs maintained graft function for at least 1 month, and 2 animals for at least 7 and 8 months, respectively. In the rats, these chambers restored normoglycemia ≥ 1 month in all of the animals, and for >6 months in 10 of the 17 STZ recipients (canine, 3/8 [38%]; bovine, 4/5 [80%]; porcine, 3/4 [75%]). In the third type of system (microreactors, diameter <900 μm), the islets were implanted into the peritoneum of spontaneously diabetic dogs (canine islets), and into STZ-induced diabetic mice and rats (bovine and porcine islets). The implants promptly reversed the diabetic state (BG <250 mg/dl) of all the recipients within 24 hours. All but 2 of the animals (1 mouse and 1 rat) sustained these levels for at least 1 month. Histologic evaluation of all three biohybrid pancreas devices (1-20 months postimplantation) revealed viable islets with granulated β-cells. These data will be compared, and some of the issues that are crucial to the clinical success of the different device systems addressed.

ENGINEERING RECEPTOR-MEDIATED CELL FUNCTIONS: QUANTITATIVE STUDIES OF CELL MIGRATION BEHAVIOR

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Development of cell-based devices for therapeutic applications will benefit from understanding key design parameters for manipulation of cell functions, such as proliferation, adhesion, migration, uptake, and secretion, in desired fashion. Such functions are generally regulated via receptor/ligand interactions, for ligands including growth factors, extracellular matrix proteins, and chemotactic attractants, so a number of the important system parameters will likely represent receptor and ligand properties.

This talk will provide a summary of efforts directed toward quantitative understanding of receptor/ligand properties governing cell migration behavior in particular, including endothelial cells, smooth muscle cells, fibroblasts, and leukocytes, all of which are involved in tissue responses to implanted materials, favorable and unfavorable.

A STRATEGY ON OPTIMAL FORMULATION FOR HEMOSOME PRODUCTION

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Experimental design approach - orthogonal array experimentation was employed in determining the optimum condition for preparation of hemosomes (Hb-containing liposomes). The optimal amount of egg phosphatidyl-choline (egg PC) was determined from experimentation to be 20 mg per ml solution. Concentration of Hb solution was chosen to be 15 g/L. Addition of egg phosphatidyl acid (egg PA) facilitated better particle size distribution; diethyl ether added would enhance the particle size distribution but, lower the encapsulation efficiency.

The optimal combination of factors and processing condition determined from such experimental design, enables us to produce Hemosomes with good particle-size distribution and high encapsulation efficiency.

IMMOBILIZED AMINOACYLASE IN STABILIZED CALCIUM ALGINATE BEADS FOR THE PRODUCTION OF *L*-PHENYLALANINE

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Aminoacylase I (EC.3.5.1.14) was immobilized by entrapment in uncoated calcium alginate beads, calcium alginate beads coated with chitosan, polyethyleneimine, polyethyleneimine-glutaraldehyde, poly-L-lysine and covalent immobilization of enzyme to calcium alginate beads, for the production of *L*-phenylalanine by the hydrolysis of a racemic mixture of *N*-acetyl-*DL*-phenylalanine. The operational stability, thermal stability, effects of pH and temperature and kinetic constants, K_m and V_{max} values of free and immobilized enzymes were studied. Scanning electron mocrographs revealed differences in the surface structures of coated and uncoated beads. The characteristics of the different immobilized systems will be discussed.

FREE AND MICRO-ENCAPSULATED *ERWINIA HERBICOLA* FOR THE PRODUCTION OF TYROSINE: KINETIC CHARACTERIZATION OF INTRACELLULAR TYROSINE PHENOL-LYASE.

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The tyrosine phenol-lyase (TPL) activity -E.C. 4.1.99.2- activity of intact *Erwinia herbicola* (ATCC 21434) cells was used to convert ammonia, phenol and pyruvate into tyrosine. The cells were grown for 24 hours in a tyrosine rich medium washed and then resuspended in a phenol (40.0 mM), ammonium (80.0 mM) and pyruvate (80.0 mM) in phosphate buffer (0.1 M, pH 8.0) solution. The cells were either used as a free cell suspension or immobilized by microencapsulation in alginate-polylysine-alginate (APA) microcapsules (800 µm). An integrated kinetic equation based on rapid equilibrium assumptions was derived and used to model the tyrosine production with time data. This model showed that if the microcapsules were agitated sufficiently the mass transfer resistance due to the Nernst layer can be reduced sufficiently to allow the reaction to be kinetically controlled. In this situation the apparent Michaelis constants for the substrates of the whole cell TPL were not significantly different whether the cells were free or encapsulated. For example at a cell loading equivalent to 1.6 mg per ml of total cell protein per ml of microcapsules the apparent K_M values for tyrosine and phenol were respectively 0.20 ± 0.03 mM and 0.5 ± 0.1 mM for both free and encapsulated cells. Under these same conditions the apparent equilibrium constant for the breakup of the TPL-ammonia-pyruvate complex to free enzyme and substrates was 4875 ± 174 mM^2 for both free and encapsulated cells. These results show that microencapsulated cells of *Erwinia herbicola* can perform as well as free cells in the production of tyrosine.

EFFECTS OF PROCESS VARIABLES ON THE RELATIVE STRENGTH AND SIZE DISTRIBUTION OF ALGINATE-POLY -LYSINE-ALGINATE MICROCAPSULES

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Alginate needs to sterilized prior to its use in the preparation of microcapsules for bio-artificial organs. The present trend is to avoid heat sterilization since it causes depolymerization. This means that one can only use lower viscosity alginates which are easily filter sterilized. It would be better to use a higher viscosity alginate which would result in stronger microcapsules. In the present study the effect of heat sterilization on the molecular weight of various alginates was investigated. The alginates depolymerised during the heat treatment. Hence after 20 minutes of heat sterilization a high viscosity alginate (MW 115,000, 3.0 % w/v in saline, pH 7.4) depolymerised to give a lower viscosity alginate (MW 70,000). While a low viscosity alginate (MW 55,000, 4.0 % w/v in saline, pH 7.4) depolymerised to give an alginate of somewhat lower viscosity (MW 45,000). The alginates resulting after various periods of heat treatment were then used to prepare alginate-polylysine-alginate (APA) microcapsules by the 2 fluid atomizer method. The microcapsules contained cells and blue dextran (MW 2.0×10^6). The core of the microcapsules was liquified by repeated treatment with citrate. The microcapsules prepared from the higher viscosity alginates, even after a heat treatment necessary to ensure sterilization, could be agitated to greater degree without breaking. This was determined by monitoring the agitation speed required to cause the release of blue-dextran (MW 2.0×10^6) from the microcapsules. These results show that a high viscosity alginate can be adequately heat sterilized and yet have chains of sufficient length to form microcapsules which are able to maintain their integrity under shear. The effect of other pertinent process variables such as airflow, liquid flow, alginate concentration and polylysine reaction time on the relative strength and the size distribution of the microcapsules were also investigated.

HEPATOCYTE ADHESION TO CARBOHYDRATE-DERIVATIZED POLY(ETHYLENE OXIDE) HYDROGELS

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Poly(ethylene oxide) (PEO) hydrogels combine the mechanical and transport properties of hydrogels with the relatively inert surface properties of PEO. We have synthesized inert PEO hydrogels, then modified them with 1-amino-1-deoxy-galactose (ADG) to create a biomaterial which interacts with the hepatic asialoglycoprotein receptor leading to hepatocyte adhesion. Hydrogels were produced by electron beam irradiation of 5-20% aqueous polymer solutions of both linear and star PEO. PEO star polymers consist of 10-100 PEO arms, each of 3,000-10,000 Da, which radiate from a divinyl benzene core. The dense packing of PEO star arms provide a greater number of derivatizable hydroxyls for a given molecular weight. We used two star varieties, one with 55 arms of 10,000 Da and a second with 43 arms of 3460 Da. The hydroxyl moieties on star and linear hydrogels were tresyl chloride activated and then coupled with a 2-4 fold excess of ligand, yielding ligand densities of 1-20 mM. Hepatocytes adhered and spread on ADG-modified star PEO hydrogels but did not spread on modified linear PEO hydrogels. Hepatocytes did not adhere to unmodified hydrogels or to hydrogels modified with 1-amino-1-deoxy-glucose, prepared as a negative control. Hepatocyte adhesion and spreading occurred within three hours of cell seeding. Albumin secretion rate, as measured via an enzyme-linked immunosorbent assay (ELISA) was used as an indicator of hepatocyte differentiated function. Cells cultured on ADG-modified star PEO hydrogels exhibited normal morphology and function. These materials are of potential interest for use in hepatocyte transplantation.

IMPLANTS CONTAINING LIVING CELLS FOR CNS THERAPY

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Biohybrid implants represent a new class of medical device in which living cells, supported in a hydrogel matrix and surrounded by a semipermeable membrane, produce and deliver therapeutic reagents to specific sites within a host. First proposed in the mid 1970's for diabetes and subsequently investigated for other metabolic and hematopoetic disorders, immunoisolation has proven a particularly advantaged route for delivery to the central nervous system (CNS) of a wide variety of naturally-occurring and recombinant secretory cell products. Treatment of refractory chronic pain by intrathecal implantation of encapsulated xenogeneic adrenal cells secreting catecholamine and enkephalins is the first of these applications to reach clinical trials. Clinical studies recently reported by Aebischer *et al* confirmed the viability, biochemical function, analgesic potential and the safe retrievability of encapsulated bovine cells in non-immunosuppressed human recipients. In addition, preclinical trials at multiple centers have demonstrated efficacy of encapsulated PC-12 cells in the treatment of chemically-induced Parkinson's symptoms for at least six months in non-human primates and for more than a year in rodents. Delivery of recombinant human nerve growth factor (hNGF) from xenogeneic producer cells has been shown to prevent lesion-induced death of choline-acetyltransferese positive basal forebrain neurons in both rodent and non-human primate protocols of the fimbria-fornix lesion model of Alzheimer's disease. Accumulating data from rodent models further demonstrates the efficacy of encapsulated cells to deliver neurotransmitters, hNGF, and other neurotrophic factors in available rodent models of Huntington's Disease and Amyotrophic Lateral Sclerosis.

MECHANICAL PROPERTIES OF TISSUE ENGINEERED CARTILAGE

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New cartilage tissue is being grown by culturing chondrocytes *in vitro* on polyglycolic acid (PGA) nonwoven scaffolds. However, one of the most important concerns in the development of new articular cartilage tissue is whether it is capable of supporting mechanical loads. We have studied the mechanical properties of the growing cartilage tissue while the PGA templates degrade and chondrocytes prolifirate and regenerate cartilage. Bovine cartilage, polymer scaffolds and tissue engineered cartilage are tested in a chamber filled with culture medium. The stress-strain behavior, stress relaxation behavior, modulus, aggregate modulus and permeability are studied. The mechanical properties are discussed in terms of polymer degradationn and tissue regeneration.

CHONDROCYTE GROWTH AND CARTILAGE FORMATION IN A THREE-DIMENSIONAL RESORBABLE MATRIX UNDER INTERMITTENT PRESSURE

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Articular cartilage provides the surface of all synovial joints with a wear resistant, lubricative pad on which the bones can glide. When this surface is severely damaged by injury or a degenerative disease, the cartilage cannot repair itself. By generating a living replacement sheet of articular cartilage, therapies such as total joint replacement may not be needed. In order to generate articular cartilage tissue a three dimensional structure is required to serve as a temporary framework for the cells as they proliferate and produce the cartilage matrix (Benya, 1978). In addition to the three-dimensional structure it is believed that mechanical loading is necessary to ensure the proper zonal arrangement of the macromolecular components, collagen and proteoglycans, into a functional matrix. In a novel bioreactor, bovine chondrocytes were seeded into a three-dimensional nonwoven mesh of polyglycolic acid and subjected to low levels (55 psig) of intermittent (5 seconds at 55 psig and 30 seconds at atmospheric pressure) pressure continuously for 7 weeks. Both the reactor samples and control incubator maintained their square shape and produced sulfated glycosaminoglycans and Type II collagen. Transmission electron microscopy revealed banded Type II collagen fibers but showed no particular arrangement throughout the depth of the tissue. The number of cells per gram of tissue in the reactor samples was consistently higher than in the control samples. The reactor samples reached a normal tissue density approximately two weeks before the samples in the incubator. The effects of different cycling regimens on the developing tissue will be discussed.

REF: Benya, Padilla & Nimni, Cell, 15:1313-1321. 1978

NOVEL DOUBLE-WALLED MICROSPHERES FOR ORAL OR PARENTAL ADMINISTRATION

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Microspheres composed of a high concentration of a powdered drug dispersed within an inert or bioerodible polymer matrix provide an attractive approach to the long term release of pharmacological agents. As the drug particles on the outer surface of the microsphere dissolve in the body fluids, tortuous micro-channels form progressively throughout the solid mass. As other drug particles come into contact with the liquid environment, they also diffuse from the microspheres to the host. This technology is not without problems, though. One drawback to this type of system is the initial "burst" effect caused by the rapid release of the drug particles trapped on the surface during manufacturing. A second problem with such systems is that a spherical form does not produce constant (zero order) drug release, due to the changes in diffusion distances in the case of non-eroding microspheres, or the changing surface area in the case of bioerodible microspheres. These single-polymer microspheres also do not lend themselves to pulsatile release of a bioactive substance, separated by long intervals of quiessence, as required by some applications, such as the delivery of vaccines. A double-walled microsphere with layers made of different polymers, inert or erodible, could solve some of these problems. The use of such polymers as oral and parental drug delivery will be disscused in the presentation.

LIFE AND DEATH OF MICROENCAPSULATED GENETICALLY ENGINEERED CELLS

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Clonal cell lines of rat embryonic mesencephalic and hippocampal origin developed by using retroviral transduction of temperature-sensitive simian virus 40 large tumor antigens were encapsulated in different biocompatible polymers. These cell lines underwent morphological differentiation at the nonpermissive temperature and in response to differentiating agents. After incorporation in biodegradable and biocompatible polymers cells remained viable for different periods of time depending on choice of the polymer, cell density per sphere and their sizes. Cell survival and cell death (apoptosis and necrosis) as well as cell replication were determined at different points of time after encapsulation. Programmed cell death was assessed by transglutaminase immunocytochemistry, nick labelling and electron microscopy. A small proportion of the dying cells showed typical morphological characteristics for apoptosis: rounded and shrunken cell bodies, condensed chromatin around the margin of the nucleus and large vesicles in the cytoplasm. As proliferation markers, both bromodeoxyuridine and 3H-thymidine were used and a MTT assay were applied for determination of metabolic activity. Large numbers of mesencephalic cells (> 80 %) encapsulated in alginate and highly viscous chitosan (68 cPas) at an optimal cell density 10^5 cells/10 uL remained viable for at least one month.
Acknowledgements: Centres of Networks of Excellence and the Parkinson's Association of Canada.

SYNTHESYS, FUNCTIONALIZATION AND CHARACTERIZATION OF POLYSTYRENE IN EMULSION FOR UTILIZATION IN IMMUNOLOGICAL ASSAY.

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Polystyrene latex with functional groups on their surface can react efficiently with antigens or antibodies. This latex in presence of suitable functional groups for covalent binding have been extensively used for immunodiagnosis and have wide applications in the field of biochemistry, colloid science, medicine and other areas. Using the method of emulsion polymerization and the report of Liu et al(*),we have obtained microspheres with narrow size distribuition (about 0,2 um in diameter) wich were functionalized with diazo groups (-N=N-). The preparation of this immunological latex consisted of the following steps: synthesys, nitration, amination and diazotization. Every step was accompained through FTIR spectroscopy and the particules size distribuition was examined in a submicrom particule analyser (Coulter) and Scanning Eletron Micrograph (SEM). Bovine serum albumin (BSA)-sensitized latex agglutinated in a few seconds,in the presence of a rabbit serum containing specific antibodies to BSA. Our experimental observations suggest that the optimization of some variables during the synthesys of the PDS latex, like the control of particules size, is very important for the success of its application in immunological assays.

REF:Liu, Dong and Zhao, J.Immunol.Methods,124:159-163, 1989

DEVELOPING AND CHARACTERIZATION OF A PLASTIC MATERIAL MADE TO OPTIMIZE THE RADIOTHERAPIC TREATMENT OF PATIENTS WITH MELANOMA AND MAMMARY CANCER.

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The radiotherapy began in the area of the ortovoltage with equipaments that produced radiation of low power penetration. Furthermore, the equipaments used to emit the necessary high doses of radiation caused reactions in the skin, thus limiting its utilization to treat mammary and skin tumor. With the objective of avoiding some undesirable effects caused by the radiotherapic treatment we have developed a polymeric material to protect the area under irradiation. The material is a platisol basically made of a vinylic resin and a well known plasticizer named di-octil-ftalate (DOP).
When it was irradiated with 7,8 Mrad (Three times the necessary dose for the therapy) the material did not present any evidence of degradation and color change. This has been confirmed by preliminary results obtained with hardness Shore A tests, FTIR and other chemical testing of the irradiated material. Others tests are been in progress and suggest that this material is suitable for our purposes.

p-NITROPHENYL CHLOROFORMATE ENHANCES THE ACTIVITY OF TRYPSIN IMMOBILIZED ON Ti-6Al-4V

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Many of the problems now seen with orthopedic implants relate to loosening of the interface between bone and orthopedic biomaterials. Because tissues ineract mainly with the surface of the implant, surface modifications are a focal point of the research on orthopedic implants. A new approach in this area is to covalently immobilize biomolecules on the surfaces with the purpose of enhancing certain cellular responses. The intent of this research was to investigate the *p*-nitrophenyl chloroformate immobilization chemistry for use with the inorganic biomaterials used in orthopedic implants. Bulk samples of Ti-6Al-4V alloy were prepared according to ASTM F86. The Ti-6Al-4V samples were activated using increasing concentrations of *p*-nitrophenyl chloroformate (*p*-NPC) according to the chemistry of Wilcheck and Miron (*Biochem. Int;* **4**:629, 1982). The number of active groups was determined by hydrolyzing the leaving groups with NaOH, which releases *p*-nitrophenylate ions producing a yellow color that can be read spectrophotometrically at 410 nm. After the samples were activated with *p*-NPC, a solution of trypsin in phosphate buffered saline, pH 7.2, was placed on the samples and incubated for 48 hours at 4°C. Concentrated urea was used to aid in the removal of trypsin that was not covalently bound to the Ti-6Al-4V. The activity of protein immobilized on the samples was quantified using the BAEE assay. Cleavage of the substrate was then measured at 253 nm using a spectrophotometer. The number of active groups created per nm^2 of nominal surface area of Ti-6Al-4V samples was: 0.79 for 2mg *p*-NPC/sample; 2.2 for 4 mg *p*-NPC; and 5.0 for 8 mg *p*-NPC. The activity of the trypsin on the samples treated with 8 mg *p*-NPC/sample averaged 9.0 BAEE units and the untreated samples averaged 8.0 BAEE units. After treatment with 8M urea, the samples derivatized with 8 mg *p*-NPC/sample showed no decrease in activity while the untreated samples showed a 67% decrease in trypsin activity. This demonstrates that activation with *p*-NPC enhances the activity of trypsin immobilized on Ti-6Al-4V.

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BIOREACTORS FOR EXPANSION OF HEMATOPOIETIC PROGENITOR CELLS

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Hematopoiesis is the regulated production of mature blood cells from stem and progenitor cells in the bone marrow. Bone marrow transplantation is used to restore hematopoietic activity in patients whose marrow has been depleted by chemotherapy or irradiation. Hematopoietic cells may be obtained from bone marrow, peripheral blood, or umbilical cord blood of matched donors and/or the patient prior to treatment. However, these sources are often inadequate, so attempts have been made to expand and/or purge (remove tumor cells) hematopoietic cells prior to transplantation. We have developed a perfusion bioreactor system for hematopoietic cell expansion that maintains constant pH, nutrient, and cytokine levels. Using this system with cord blood mononuclear cells on a feeder layer of irradiated bone marrow stromal cells, we expanded CFU-GM (granulocyte-macrophage colony-forming unit) numbers by 20-fold, and LTC-IC (long-term-culture-initiating cells; most primitive progenitors that can be assayed in culture) numbers by as much as 3-fold. CFU-GM and other mature progenitor cells are required for rapid engraftment, while LTC-IC provide sustained engraftment. We have recently developed bioreactor culture chambers that retain progenitor cells in the absence of a stromal cell feeder layer, and have demonstrated equal (20-fold) expansion of peripheral blood CFU-GM numbers with or without a stromal layer. Elimination of the stromal layer simplifies the culture system, shortens the time required to establish active hematopoiesis, and eliminates the need for a bone marrow donor for transplants of cord or peripheral blood progenitor cells. Besides providing an environment conducive to progenitor cell expansion, perfused bioreactors are superior to bag or flask cultures because they minimize chances for contamination during feeding, are easier to scale up, and are inherently superior for GMP compliance.

LARGE SCALE IN VITRO CULTURE OF TISSUES FOR TRANSPLANTATION: FROM THE LABORATORY TO THE MARKETPLACE

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Advanced Tissue Sciences (ATS) integrates the technologies of polymer chemistry, cell biology and biochemistry to create physiological tissues in vitro. The patented core technology is based on seeding stromal cells onto three dimensional scaffolds and mimicking in vivo growth conditions to support cell growth and secretion of matrix proteins and growth factors to create tissue substitutes. Using this technology, we have successfully replicated a variety of human tissues including skin, cartilage, bone marrow and liver.

The Dermagraft family of products, our most advanced products, are designed for use in the treatment of severe burns, chronic skin ulcers, and reconstructive and cosmetic surgery. The products are composed of neonatal fibroblasts and extra-cellular matrix proteins in a three dimensional support. We are currently in pivotal clinical studies for burns and diabetic ulcers and a feasibility trial for pressure ulcers. In addition, we have filed an Investigational Devise Exemption with the FDA for Dermagraft Transitional Covering, for use in burn injuries as a replacement for human cadaver skin.

The Dermagraft family of products will be produced using a common manufacturing process. A modular, automated, closed system has been developed to manufacture highly reproducible tissue in a sterile environment. Extensively tested cells from our working cell banks are injected automatically into tissue bioreactors and a computerized system regulates growth conditions during the manufacturing period. Monitored growth conditions include pH, CO_2, glucose utilization and ascorbate levels. Tissues are frozen, quality controlled for matrix properties and cell viability, then shipped and stored in the same bioreactors for clinical use. This system allows for flexibility in manufacturing, upscaling and expansion to additional sites along with a cost effective product.

Engineered human cartilage is ATS's next product heading for clinical trials. We have successfully replicated cartilage tissue in vitro and in vivo, and have performed collaborative preclinical studies for articular resurfacing. Automated bioreactor systems have been developed for the reproducible manufacture of the cartilage tissue. Initial resurfacing, facial reconstruction, meniscal repair and replacement, and treatment of osteoarthritis.

ATS is developing tissue engineered liver that could provide a bridge to transplantation. We have cultured enzymatically active animal and human liver cells in long-term cultures. Preclinical trials are ongoing to determine if such constructs can replace certain aspects of liver function.

Such tissue engineered constructs have the potential of contributing to the field of transplantation and alleviating tissue shortage problems.

MECHANISTIC STUDY ON TOXICITY OF POSITIVELY CHARGED LIPOSOMES CONTAINING STEARYLAMINE (SA) TO BLOOD COMPONENTS.

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Positively charged liposomes have been reported as effective trappers of negatively charged drugs, however those containing SA have cytotoxicity. We studied the interaction (fusion and/or lipid transfer) of SA liposome with erythrocyte ghost (EG) or platelets by resonance energy transfer assay. Interaction of SA liposomes with EG or platelets, dependent upon the conc. of SA, was observed in 5 mM Hepes buffer, however, in 5mM Hepes/150 mM NaCl, it was remarkably inhibited. In the presence of carboxymethyl chitin (CM-chitin), SA liposome-EG interaction was inhibited, indicating that CM-chitin reduces the tendency of SA liposome to interact with EG. SA liposome-platelet interaction was not affected by CM-chitin or phagocytosis inhibitors: EDTA, cytochalasin B, or 2,4-dinitrophenol and iodo acetate, indicating that SA liposome-platelet interaction involves the fusion and/or lipid transfer between SA liposomes and platelets, and they are controlled mainly by electrostatic interactions between SA and glycoproteins on the surface of platelets.

REF: Nishiya & Lam, Colloids and Surfaces B: Biointerfaces, 1: 213-219. 1993.

STUDY OF IN VITRO STABILITY OF POLYMERIZED LIPOSOMES

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Liposomes have been extensively explored as carriers for improving the delivery of various therapeutic drugs. Although they are principally administered parenterally, they can also be used for the oral administration of drugs. We studied the stability of liposomes composed of polymerized phospholipid, 1,2-bis-(octadeca-2,4-dienoyl)-sn-glycero-3-phosphocholine (DEPC) and cholesterol (CHO), to pH changes, the presence of bile salts and the repetitive freezing and thawing, monitoring the leakage of ^{3}H-sucrose entrapped in liposomes. The stability of polymerized DEPC /CHO liposomes to acidic media (pH 3 and 2), the presence of bile salts (20 mM) and the repeated freeze-thaw, was much higher than that of nonpolymerized DEPC/CHO liposomes and liposomes composed of conventional phospholipids. The stability of polymerized DEPC/CHO liposomes was increased in the presence of carboxymethyl chitin, which stabilizes the liposomes against the plasma protein (Nishiya & Ahmed, 1990). These results suggest that polymerized DEPC/CHO liposomes may be able to use for oral administration of drugs.

REF: T. Nishiya and S. Ahmed, J. Biochem. 107: 217-221. 1990.

IMMOBILIZED RHODANESE: A SIMULAR MODEL FOR THE STUDY OF CYANIDE DETOXICATION.

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Nitrite and thiosulfates are always co-administered to produce a synergic antidotal effect in the treatment of cyanide poisoning. The former has a methemoglobin forming ability, while the latter serves as a co-substrate of the enzyme Rhodanese(thio sulfate: cyanide sulfurtransferase EC 2.6.1.1). Recent reports have shown that the rhodanese enzyme is inactivated in vitro by nitrite ion (Alexander et al 1989). This finding could affect decision making in cyanide poisoning chemotherapy.

Immobilized Rhodanese represents near model a system for studies of cyanide detoxication and ion effect. Rhodanese was extracted from Rat Liver and immobilized on calcium-alginate gels. The immobilized enzyme had a pH optimun of 7.35±0.12 : ., Km values of 3.42±0.16 mM and 1.22±0.13 mM for $S_2O_3^=$ and KCN respectively. The enzyme was inhibited competitively by $NaNO_3$ and activated by Ca^{2+}, Zn^{2+} and Cu^{2+} (4.10^{-4}M)

Cyanide extracts prepared from cassava tissue (Nok et al 1993) at concentrations of about 100 ppm were copletely converted to SCN^- at differencial periods in the presence and absence of the inhibitor and the individual activators.The observed periods were 88±3 min in the presence of the inhibitor, 58±4 min with any of the divalent cations and 70±6 min when the inhibitor was incubated alongwith a given divalent cation.

These findings could be of immence relevance in future pharmacological research in terms of active drug ingredients in the management of cyanide poisoning.

REF: Alexander et al,J.Biochem.Toxicol,4:29-33.1989
Nok et al,J.Fd.Biochem,16:109-118.1993

IMMUNOADSORPTION APHERESIS OR/AND DOUBLE FILTRATION PLASMA PHERESIS FOR AUTOIMMUNODISEASES BEING RESISTANT TO THEIR MEDICATION THERAPIES

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This study has a purpose of usefullness of immunoadsorption apheresis or/and double filtration plasma pheresis for autoimmunodiseases showed resistant to their medicaion therapy.

From April of 1988 to the end of 1993, 77 patients of the autoimmunodiseases were treated with immunoadsorption apheresis or/and double filtration plasma pheresis. They included nerve and muscle system as 8 of Myasthenia grevis and 7 of Guillain-Barre syndrome, skin diseases as 4 of Pemphigus and 4 of Pemphigoid, kidney diseases as 12 of Systemic lupus erythematousus and 2 of focal glomerulosclerosis, and others as 1 of anticardiolipin-antibody syndrome and 1 of cold hemoagglitinin syndrome. For this series, Japanese immunoadsorber and special second filter were used. The volume of treated plasma were from 2,000 to 4,000 ml in one apheresis with average 60 ml per minute of blood flow.

The removal ratio of IgG was from 13 to 28% by immunoasaorption apheresis, but it was 23 to 47% by the second filter. The removal of IgM, however, was from 19 to 33% by immunoadsorption apheresis, and from 32 to 50% by the second filter.

98% of all were recovered from their clinical symptomes by these apheresis and came back to their ordinary lives. Several cases of them were returned to intermittent apheresis in once or twice a month, but they were rehabiliteted with these apheresis.

In the point of view, immunoadsorption apheresis has much less chance of albumin infusion during or after apheresis than double filtration plasma pheresis.

CLINICAL APPLICATION OF PLASMAPERFUSION IN MYASTHENIA GRAVIS

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At present several immunologic diseases can be treated with Plasmaperfusion (PP) by specific adsorbent materials. We employed PP treatments in myasthenic patients with high levels of anti acetylcholine receptor antibodies (AChR abs) and investigated the relationship between clinical results and specific removal. Really pathogenetic anti AChR abs seem to be correlated to IgG1 and IgG3 subclasses. In order to analyze the turnover of IgG subclasses during PP, we selected 11 patients affected with M.G., unresponsive to thymectomy and steroid treatment (III-IV class of Osserman's classification). The treatment was performed with Plasmaflo AP-05H plasmaseparators and Immusorba IM-T350 columns (Asahi Med.Co.). Every cycle consisted of 6 PP sessions carried out every 2-3 days. Both clinical and electrophysiological improvements were observed in all patients. The serum concentration of immunologic markers, before and after each PP session, showed the following decrease (mean % ± SD): IgG 25.7±12.7, IgA 20.4±11.3, IgM 23.2±11.4, C3 36.8±10.1, C4 42.7±14.8. The serum concentrations of IgG subclasses, reported as % of total IgG, before the first treatment were: IgG1 63.5±10.1, IgG2 28.2±11.3, IgG3 5.2±2.0, IgG4 3.0±1.4. After the treatment they presented the following decreases (mean % ± SD): IgG1 25.8±17.6, IgG2 32.4±12.0, IgG3 31.1±10.7, IgG4 20.3±13.4, while the anti AChR abs decreased of 39.4±18.0. After each PP treatment there were increases of both IgG (32.0%±23.7%) and anti AChR abs (63.5%±82.7%). In our experience we conclude that anti AChR abs decrease is higher than total IgG decrease, and that removed abs are, probably, part of IgG1 subclass. Dosage of specific anti AChR abs subclasses, which is now in progress, will probably improve our knowledge about this intriguing problem.

RECENT ADVANCES IN MUCOADHESIVES FOR DRUG DELIVERY

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The interaction of aqueous solutions and gels of submaxillary gland mucin with various homo- and copolymers of acrylic and methacrylic acid can be quantified by flow detachment studies as well as by near-field FTIR and attenuated total reflectance IR spectroscopic investigations. Such studies provide a classification of novel bioadhesives according to the strength of the bioadhesive bond, as well as analysis of the importance of the chain interpenetration mechanisms in the overall bioadhesive behavior.

ATR-FTIR spectroscopy was developed for investigation of chain interpenetration at a PAA-mucin interface. A thin film of acrylic acid, polymerized below the gelation point, was contacted with a buffered mucin solution and the ATR-FTIR spectrum was collected *in situ* as a function of time. The experimental results show evidence in support of chain interpenetration, indicate that PAA and mucin are compatible under physiological conditions, and prove that adhesion is limited by the extent of chain interpenetration across the biointerface.

The adhesive force was also determined from flow characteristics of bioadhesive microparticles and correlated to their chemical structure, especially the carboxylic groups on the particles.

This work was supported by the National Institutes of Health.

POLYMER BASED SPECIFIC SORBENTS

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We prepared polyhydroxyethylmethacrylate (PHEMA) beads, in the size range of 150-250 μm by suspension polymerization. The hydroxyl groups on the PHEMA beads were activated by cyanogen bromide. Then, several ligands were covalently attached through the active points for different purposes. Heparin molecules were immobilised onto the PHEMA beads to improve their biocompatibilities. Albumin conjugation to the covalently attached heparin was evaluated for further passivation of the surfaces. Low molecular weight heparin was immobilised onto the PHEMA beads as a ligand for cholesterol adsorption from both hypercholesterolaemic human and rabbit plasmas. Protein-A and DNA immobilised PHEMA beads were investigated for specific sorbent hemoperfusion for removal of antibodies. PHEMA and its copolymers in the bead form were evaluated for immobilisation of cells. BHK and MDBK cells were cultured on these beads. Collagen and fibronectin molecules were covalently attached to PHEMA beads to improve hepatocyte immobilisation for possible artificial liver support system. Polymeric sorbents based on PHEMA, polyfluorostyrene and their modified forms were utilised for separation of intestinal epithelial cells from fibroblasts. This presentation give an overview of our related studies mentioned above.

GENETICALLY ENGINEERED *E. COLI* DH5 CELLS CONTAINING *K. AEROGENES* GENE MICROENCAPSULATED IN ARTIFICIAL CELLS FOR REMOVAL OF PLASMA UREA AND AMMONIA.

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Microencapsulated genetically engineered *E.coli* cells can efficiently remove urea and ammonia (Prakash and Chang, 1993). We have carried out further studies. Alginate concentration of 2.0 w/v, liquid flow rate of 0.0724 ml/min and air flow rate of 2.0 lit/min are found to be the best conditions to make the microcapsules of 500 ± 45 μm diameter. They are mechanically stable up to 210 rpm agitation. The in-vitro plasma urea and ammonia removal efficiency by encapsulated and free bacteria is evaluated. The results shows that a 100 mg alginate-polylysine-alginate encapsulated log phase bacteria can efficiently lower 92.13 % of the plasma urea within 20 minutes compared to 97.46 % by free bacteria. Also the same amount of the encapsulated bacteria can lowers plasma ammonia from 975.14 ± 70.15 μmol/L to 81.151 ± 7.37 μmol/L in 30 minutes. There are no significant differences in the depletion profiles by encapsulated or free bacteria for the urea and the ammonia removals. In this study, for the first time, we shows the feasibility of a novel approach to remove plasma urea and ammonia using microencapsulated genetically engineered *E.coli* cells. The details of the kinetics of the above studies will be discussed.

Ref. : Prakash, S. and Chang, T.M.S. (1993). Biomat. Arti. Cells and Immob. Biotech. 21(5), 629-636.

MICROENCAPSULATION OF ISLETS WITH SEMI-PERMEABLE POLYMER MEMBRANES: ENCAPSULATION TECHNOLOGY AND POLYMER SCREENING

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The microencapsulation of cells with semi-permeable polymer membranes is a promising development for the treatment of several hormone deficient diseases. It appears that the most likely initial application of such technology will be in the treatment of diabetes through the encapsulation of islets of Langerhans. This will involve the formation a bio- and cell-compatible polymer membrane which is relatively non-degradable and is homogeneously formed into an optically smooth geometry. Such microcapsules, once implanted, would function much as a natural pancreas does, regulating insulin in response to changes in the glucose level. Prior to any animal testing several polymer, membrane and capsule parameters must be optimized. These include the cell viability in the presence of polymers, membrane porosity and permeability, capsule smoothness, transparency, size dispersity, strength and durability, as well as the insulin response of the capsules. We will discuss results on an extensive polymer screening (> 1000 combinations of polyanions and polycations) of capsules prepared via a complex coacervation reaction. These screening tests have been performed in both static beaker tests as well as with novel technologies which relax some of the fluid mechanical and polymeric time constraints which are inherent to the formation process of microcapsules. This has enabled a larger number of polymer combinations to be tested, including several materials which would be difficult to form into useful capsules using traditional impact technologies. The correlation of polymer properties with membrane structure will also be presented, as will the problem of islet centering within the capsule.

ENZYME-BASED LIVER SUPPORT SYSTEMS

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The design and optimization of enzymic systems for liver support require appropriate analytical models. In this report, two reactors are considered: a)Single Enzyme Hollow Fiber (SH) Reactor: In this system the enzyme, in soluble form, is in the shell section of the unit and catalyzes a two-substrate process . This reactor can also be used for one-substrate processes when the enzyme is inhibited by a product, as well as for one-substrate reversible processes. In all cases, substrates and products diffuse across fibers wall. We also consider that backmixing takes place in the lumen section of the unit and that the solution in the shell section is perfectly mixed. b) Dual Enzyme Multi Layer (DM) Reactor: This is of the packed bed type . The two enzymes are immobilized on insoluble carriers set in alternating layers and catalyze two consecutive reactions. For each reactor, a model was written in terms of the compartmental analysis. These models account for a variety of kinetic as well as mass transport processes, and were used for extensive numerical simulations. The calculated results clearly point to some of the most critical characteristics of the systems considered. Thus,depending on operational conditions employed, the SH reactor is controlled by kinetic processes, diffusion processes or both. As for the DM system and when enzyme inhibition is or is not operative, reactor performance depends to a large extent on its physical configuration, particularly on the number of layers among which the two enzymes are distributed. The theoretical analyses described above were subsequently supplemented by a large body of experimental studies on appropriate model systems. In these studies, Tryptophanase was used in an SH-type reactor, while immobilized Glutathione-S-Transferase and γ-Glutamyl Transpeptidase were used together, in a series of DM reactors of various configurations. The experimental results obtained with these systems could be assessed in terms of the analytical models developed. As such, these models proved to be useful, reliable and instructive tools for interpreting the experimental results, as well as for establishing the optimal reactors configuration and mode of operation.

BIOCOMPATIBILITY OF CARDIOVASCULAR MATERIALS

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Biomaterials and medical devices are now commonly used in cardiovascular medicine and surgery. Most implants serve their recipients well for extended periods by alleviating the conditions for which they were implanted. However, some develop complications which constitute device failure, necessitating reoperation, or causing death or serious disability. Complications are largely based on biomaterial-tissue interactions that include the following: 1) *local biological effects* such as blood-material interactions (e.g., blood platelet adhesion and activation, complement activation and hemolysis), toxicity, modification of normal healing (e.g., encapsulation, foreign body reaction and pannus overgrowth), infection and tumorigenesis; 2) *systemic and remote effects* such as embolization of thrombus or biomaterial hypersensitivity, elevation of unusual elements in blood and lymphatic particle transport and 3) *effects of the host on the implant*, such as physical/mechanical effects and biological degradation processes (e.g., absorption of substances from tissues, enzymatic damage, calcification). The importance and mechanisms of these interactions in heart valve prostheses, vascular grafts and other cardiovascular devices will be reviewed and the current status of efforts to prevent thrombosis, calcification and aberrant healing, the most frequent causes of device failure, will be discussed. Emphasis will be placed on data generated by evaluation of retrieved experimental and clinical devices.

REF: Schoen FJ, Levy RJ and Piehler HR, Cardiovasc. Pathol. 1:29-52, 1992

CELL IMMOBILIZATION IN HYDROGEL MATERIALS

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Cell entrapment within hydroxyethyl methacrylate-methyl methacrylate (HEMA-MMA) microcapsules is expected to facilitate the transplantation of cells by providing immunoprotection, thus avoiding the need for immunosuppressive drugs. Such capsules are potentially useful as controlled release devices with natural or genetically engineered cells for the treatment of diabetes, Parkinson's disease and a wide variety of other disorders (e.g., gene therapy). Our studies are directed to understanding the interrelationship among capsule properties, the tissue reaction and the behaviour of the encapsulated cells. For example, HEMA-MMA microencapsulated xenogeneic (human HepG2) hepatoma cells, used as models for hepatocytes, within a Matrigel/α-MEM core, have been implanted into various tissue locations. There has been a well vascularized tissue responses to capsules associated with omental tissue in the OM and IP embedded locations and also in the SC location, as is desired for enhanced nutrient delivery and product absorption. Also foreign proteins on the capsule surface and the intracapsule extracellular matrix component affect the tissue response. Characteristics related to biocompatibility have also been measured: capsule surface chemistry, nature of adsorbed protein and the extent of *in vitro* macrophage activation.

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AN APPROACH OF TARGETING CELLS WITH BIOSPECIFIC FUNCTIONAL POLYMERS INCORPORATED IN VESICLES

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Smooth muscle cells are known to be sensitive to heparin and soluble heparin-like dextran derivatives (Letourneur et al., 1993). However, their precise mechanisms regarding the cell proliferation and intervention of a specific membrane receptor are still under investigation. An approach to this problem lies in the use of vesicles as vehicles of either soluble active compounds encapsulated within the internal volume or compounds grafted to phospholipids and incorporated in the vesicle bilayer. To avoid a potential disruption of the cell membrane when in contact with vesicles, the lipid and fatty acid composition of rat smooth muscle cells and a human endothelial cell line (EA.hy 926) were determined by gas chromatography. In addition, these characterizations were performed at different cell density in presence or absence of soluble polymers. Vesicles based on phosphatidylcholine / phosphatidyl-ethanolamine / cholesterol (72.9 / 16.5 / 10.6 mol%) exhibit a composition similar to the targeted cells. These vesicles were prepared by sonication and characterized by liquid chromatography on a gel exclusion column using both turbidimetry and refractive index detections. The evolution of the aggregate size (initial mean diameter = 70 nm) was followed as a function of time, for vesicles stored at different temperatures. Moreover, the membrane permeability was assayed by measuring the turbidity at 350 nm during vesicle solubilization by octyl glucoside, a non-ionic surfactant. From the obtained results, bilayers of sonicated vesicles exhibit a rather good stability with time at 37°C, making them good candidates as drug delivery systems. Encapsulation in such vesicles of heparin and heparin-like derivatives is in progress.

REF: Letourneur, Logeart, Avramoglou & Jozefonvicz, J. Biomater. Sci. Polymer Edn, 4:431-444. 1993

INTRACORPOREAL BIOREACTOR WITH IMMOBILIZED PHOSPHOLIPASE A_2 TO REDUCE PLASMA CHOLESTEROL

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An elevated concentration of plasma cholesterol is a critical factor contributing to the development of atherosclerosis and heart disease. A proposed treatment for chronic high cholesterol involves an implantable polymeric hollow fiber encasing phospholipase A_2 (PLA_2). This enzyme facilitates the removal of cholesterol by hydrolyzing the phospholipids' *sn*-2 fatty acyl ester bonds, allowing modified-LDL to be taken up by the liver at an accelerated rate. Because PLA_2 is non-specific in reacting with phospholipids, the enzyme is immobilized onto beads to prevent leaching from the bioreactor's pores. Measuring 1.1 mm in diameter and 50 mm in length, the hollow fiber is constructed to selectively permit low density lipoproteins (LDL) to enter into the lumen and react with PLA_2. The goal of this *invivo* study was to characterize the effect of the PLA_2-filled bioreactor on the cholesterol level of hypercholesterolemic New Zealand white rabbits when implanted for 50 days. Bioreactors were constructed by first immobilizing the enzyme to beads with amino residues and packing the beads with the enzyme into the hollow fiber. The bioreactor's enzymatic activity was found by titrating the reaction between PLA_2 and the substrate L-alpha-phosphatideylcholine dipalmitoyl with sodium hydroxide. Cholesterol data from rabbits indicate that the bioreactor, when containing a sufficient enzymatic activity, is able to successfully slow the elevation of plasma cholesterol in rabbits maintained on a high cholesterol diet. Blood tests have shown that the treatment causes no change with respect to liver function or blood composition.

TREATMENT OF SEVERE DRUG INTOXICATION USING DIACETYLCHITIN-COATED RESIN HEMOPERFUSION

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We have developed a clinical hemoperfusion system using neutral macroreticular styrene-divinyl benzene based resin (surface area=600 M^2/g, average porosity of 85 Å) coated with diacetylchitin (DAC) for improved hemocompatibility. Preclinical animal studies demonstrated that DAC-resin preparations were pyrogen-free, non-toxic and non-hemolytic. White blood cell and platelet counts decreased by 12 and 16% respectively with DAC-resin as compared to 65 and 60% with the uncoated resin, in trial hemoperfusion in the dog.

Fifteen patients with severe drug intoxication were treated with DAC-resin hemoperfusion. The causes of intoxication were fatal doses of hypnotics, sedatives and tricyclic antidepressants such as phenobarbital, amobarbital, pentobarbital, valium, chlorpromazine, amitriptyline, carbamazepine, and doxepin. The length of intoxication averaged 26.3 hr (range: 3.5 - 72 hours). Before hemoperfusion was begun, all patients were in deep coma and did not respond to conventional emergency therapies including intravenous fluids, tracheostomy and assisted ventilation, hemodialysis, anti-shock drugs, central nervous system stimulants, etc. All but one patients suffered from a decreased or undetectable blood pressure. In eleven patients, drug concentrations were determined and 2 hr hemoperfusion reduced the circulating drug by an average of 48.6% (range: 15-94%). Five patients recovered consciousness within 2 hr of hemoperfusion and responded to questions. All other patients showed improvement in their state of consciousness within 6 hr of hemoperfusion. One patient relapsed into coma due to a rebound of circulating drug concentration caused by release of drug from the adipose tissue. This patient received a second hemoperfusion 24 hr after the first one and fully regained consciousness. All patients survived and were discharged within 3-4 days of hemoperfusion treatment. In summary, our clinical results demonstrate that DAC-resin hemoperfusion is an effective and safe therapy for the acute treatment of severe intoxication due to hypnotics, sedatives and antidepressants.

TREATMENT OF CHONDRAL DEFECTS WITH CULTURED AUTOGENOUS ARTICULAR CHONDROCYTES: A CANINE MODEL

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Recent human trials have utilized autogenous periosteum, perichondrium and cultured articular chondrocytes to repair osteochondral defects with promising results. We have developed a canine model in order to determine the optimal conditions for articular cartilage regeneration. Preliminary studies have used autogenous cultured articular chondrocytes, suspended in media, and injected under periosteal flaps. Two chondral defects (4mm diameter and 1mm depth) were made with a dermal punch in the intercondylar notch of the right knee of adult canines. The defects were 1 cm apart and approximately 2 cm distal to the joint space. Periosteal flaps were excised from the proximal tibia and sutured to the intact cartilage to enclose the defects. Chondrocytes previously retrieved from non-articulating sites in the contralateral joint and grown *in vitro* for 3 weeks were injected under the periosteal flap at a cell density of $2x10^6$ cells/defect. Fibrin glue was used to seal the periosteal flap. Cell-free defects, with or without periosteal flaps, served as controls. The operated knee was immobilized by external fixation for ten days after which the animals were allowed to ambulate freely. Euthanasia was performed six weeks after surgery and the defect sites retrieved and processed for immuno-histochemistry and standard histologic analysis. Sagittal paraffin sections were stained with H&E, Safranin-O, and with antibodies to Type I and Type II Collagens and Link protein. At six weeks the control defects were filled with a fibrous layer containing fibroblast-like cells. The tissue within the defects tested positive for Type I and negative for Type II collagens. The cell-implanted defects contained cells with the morphology of both fibroblasts and chondrocytes. The chondrocytes were observed at the defect interfaces and appeared to be integrated with the extant cartilage matrix. The superficial layer and central core of the defects were composed of fibroblasts. These initial results indicate that the canine model provides a means to assess the chronology of articular cartilage regeneration and the effects of various interventions on the process.

THE BIOCOMPATIBILITY OF THE IMMUNO-ISOLATED MEMBRANE MICROCAPSULES FOR ISLETS IMMOBILIZATION

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The problems of immune rejection continue to be the major obstacle of islet transplantation as a treatment for type I diabetes. A possible solution is to protect the transplanted islets from the host's immune system by means of semi-permeable immuno-isolated membrane. We have already prepared a new membrane, and in vivo studies, it can protect rejection during xenotransplantation. This paper is to study the biocompatibility of the microcapsules for islets immobilization. The microcapsules were made of calcium alginate and poly-L-lysin, and the diameter was about 1-0.8 mm in size. Two monkeys and 18 rabbits were used. Under sterile condition, 8 ml microcapsules were transplanted into the abdominal cavity of monkey, and 3-5 ml microcapsules into the rabbit. The abdominal caivty was opened after one year of transplantation in monkey, and half to one year in rabbits. In monkey, the peritoneal membrane was very smooth and no adhension, while there was no pathological changes in liver and kidney and the funtions were all normal. Those were same as in rabbits. There was no foreign body reaction. It shows that the microcapsules which we made of have good biocompatibility and it is very safe as transplanted into the diabetic patients.

REF: Shu & He, J.Biomat.Artif.Cells,Immob. Biotech. 21:85-89,1993

THE EFFECT OF GROWTH FACTOR ADSORBED ON ECM COATED AMMONIA PLASMA MODIFIED PTFE AND ePTFE ON THE GROWTH OF HUVEC AND HSVEC.

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The poor patency of artificial vascular may be accounted for the fact that the endothelialization of implanted synthetic grafts does not occur in humans. We have employed an ammonia plasma modification technique to alter the surface chemistry of PTFE and ePTFE. These surfaces have shown increased binding to ECM components and high performance towards HUVEC adhesion and growth (1,2). Endothelial cell growth supplement, ECGS was immobilized on ECM coated control and plasma treated surfaces in order to provide localized and persistent stimulation to seeded HUVEC and HSVEC. The seeded HUVEC and HSVEC on various surfaces were cultured employing two type of culture medium to investigate the synergism between soluble ECGS in the medium and immobilized ECGS. Enhanced growth of HUVEC and HSVEC 6 days after cell seeding was observed when ECGS was present in the immobilized and soluble form.

Ref: 1. Sipehia, R., Biomat. Art. Cells & Immob. Biotech., 21(5):647-658(1993).
2. Sipehia,R. et al,ibid,21(4):455-468(1993).

RABBIT AND HUMAN CORNEAL EPITHELIAL CELLS ADHESION AND GROWTH ON IMMOBILIZED EFG, ECM COMPONENTS AND EGF/ECM ON AMMONIA PLASMA TREATED POLYMERIC LENTICULES.

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The advantages of utilizing synthetic inlays for the replacement of diseased or opaque cornea are manifold. However, re-epithelialization of synthetic inlays may present a major obstacle. Immobilization of EGF and ECM proteins were carried out on control and ammonia plasma treated lenticules made from P(HEMA) and silicone polymers. Adhesion and growth of rabbit corneal epithelial cells and growth of human corneal epithelial cells were studies on several surfaces. At two hours after epithelial cells seeding of various lenticules, enhanced cell attachment on plasma treated lenticles was observed. An improved growth of cells on lenticules prepared from plasma modified Silicone polymers and P(HEMA) was reflected in the amount of tritium uptake ($p < 0.001$). Light microscopy study showed the improved rabbit and human corneal epithelial cells coverage of EGF, ECM, EGF/ECM adsorbed plasma modified inlays.

MEMBRANE BASED ARTIFICIAL ORGANS

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The development of membrane-based systems as artificial organs has recently made significant progress in establishing their validity as potential alternatives for the treatment of many diseases. Since the early seventies, semipermeable hollow fiber membranes have been utilized to culture and maintain a wide variety of transformed and primary cells. Currently, two important areas of interest are the artificial pancreas for the treatment of insulin dependent diabetes and the liver-assist device for the temporary treatment of acute liver failure. Several types of devices have been designed to sequester the cells from the bloodstream, yet provide bidirectional transport for cell nutrients, substrates, wastes, and key metabolites. The long term patency of an intravascular artificial pancreas device has been demonstrated to be similar to that of current vascular graft implant. The implantation of porcine islets in pancreatectomized dogs has been carried out in these intravascular membrane-based devices providing up to 30 units of insulin per day or 75% of the animal's daily insulin requirement for nearly six months. In other studies, hollow fiber cartridges containing isolated mammalian hepatocytes have been used in an extracorporeal circuit and significantly prolonged the survival of animals with drug-induced acute liver failure. In preliminary experiments using similar systems, porcine hepatocytes have been used successfully to provide temporary therapy for patients in acute liver failure awaiting a liver transplant. These results demonstrate the unique characteristics of hollow fiber membranes in artificial organs which can be successfully utilized to transplant xenogeneic tissue in highly discordant animals, without the need for immunosuppressive therapy.

CONTINUOUS PRODUCTION OF CEPHALOSPORIN-C BY *Cephalosporium acremonium* CELLS IMMOBILIZED ON VARIOUS MODES

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The advent of immobilized whole cell technology has generated considerable interest in the field of biotechnology. Use of immobilized fungal cells, has been observed to control effectively the rheology of the mycelial broths. Siran carriers, a commercially available catalyst support, was used successfully in the batch and continuous production of Cephalosporin-C by mycelial cells of *Cephalosporium acremonium*. The productivity of an 1.2 l, 3-phase air lift bioreactor using Siran carriers, was compared directly with the pellets and silk sachets form of immobilization $CPC_{production}$ in ALR. The specific growth rate of $0.47h^{-1}$ and $0.35h^{-1}$ were observed with Siran and Silk respectively. However, the specific antibiotic production rate of immobilized cells were at 125% and 140% at 200h (Pellets 100%) for Silk and Siran respectively. However, the 3-phase fluid dynamics of the broth were well controlled and exhibited enhanced O_2 mass transfer and improved viscosities. Results are discussed in terms of the potential for using Siran in industrial bioreactor system.

STUDY OF FILTER USED FOR LEUKOCYTE REMOVAL FROM RED CELLS

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White cells(WBC)-poor blood components have been advocated for transfusion to prevent,decrease,or delay alloimmunization and to reduce the risk of transfusion-transmitted virus diseases,graft-versus--host disease,nonhemolytic febrile transfusion reactions, and immunosuppression.Several simple and effective procedures have developed to prepare WBC--depleted blood products. So far,filtration using specific WBC depletion filter is the best method of preparing WBC-poor red cell(RBC) concentrates. In this paper, the efficiency of 6 kinds of filter materials (polyester fiber,cotton wool,cellulose acetate, polyvinyl alcohol fiber,vinylon fiber and co-woven fiber) specific for leukocyte depletion from red cell was evaluated respectively. The data demonstrated that co-woven fiber was most effective in the removal of leukocyte, followed by vinylon fiber. The leukocyte depletion capacity and propriety of co-woven fiber designed for removing leukocyte from whole blood or red cell concentrates(RCC) were studied systematically by counting leukocytes and measuring red blood cell volumes. The datd presented in this study showed that the efficiency of leukocyte removal was improved as the amount of used fiber increased.A column filled with 20-gram co-woven fiber could remove 99.9-99.99% of leukocytes from whole blood or RCC. Residual leukocytes did not exceed 5.0×10^6 and red cell recovery was more than 90%, which was higher than that of Imugard filter.

REF: R.N.I.Pietersz,Comparison of Five Different filters for the removal of leukocytes from red cell concentrates,Vox Sang,62:76-81.1992

ENGINEERED LIVER TISSUE: STRUCTURE AND FUNCTION OF NORMAL RAT HEPATOCYTES MAINTAINED IN A HOLLOW-FIBRE BIOREACTOR

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Introduction The early clinical experience of a number of groups using extracorporeal liver assist devices (ELAD) based on hepatocytes or hepatocyte-derived cell lines cultured in hollow fibre bioreactors, indicates that such devices can provide metabolic support to patients with hepatic insufficiency. We are interested in the nature of the "engineered tissue" formed by normal (untransformed) hepatocytes when cultured on hollow fibres, as a basis for understanding and optimising ELAD performance. Methods Bundles of 150 cellulose acetate hollow fibres (Duo-Flux, Althin CD Medical Inc.) were potted in a Silastic medical elastomer (Dow Corning Corp.) and placed in a bioreactor shell (Vitafiber I, Amicon Corp.). Hepatocytes were isolated from male Wistar rats by collagenase perfusion. Approximately 500 mg of cells were injected into the extracapillary space of the reactor. The reactor was perfused with a serum-free, hormonally-defined medium via the intracapillary and extracapillary fluid paths, and the perfusates assayed for evidence of liver function. After 1-5 days the culture was fixed and processed for transmission electron microscopy. Results Biochemical estimates of cell viability (reflected by accumulation of the enzymes AST, ALT, and LDH in perfusates) and hepatocyte function (including urea synthesis rates and albumin synthesis and accumulation in perfusates) suggest that a functional hepatocyte mass is stably maintained in the reactor for at least 5 days. Morphologically there is cell-fibre and cell-cell attachment with the formation of multilayered cell masses on the exterior fibre surface. The ultrastructure of the cells in these masses shows features of normal hepatocytes. Conclusions In this hollow-fibre culture system, isolated rat hepatocytes form a multicellular, three-dimensional organization in which the cells show structural and functional features of normal hepatocytes. This simple system provides a basis for further study of the biology of engineered liver tissue.

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HEAT-STABILIZED MICROSPHERES AS A SUSTAINED DRUG DELIVERY SYSTEM FOR THE ANTIMETABOLITE, 5-FLUOROURACIL

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Artificial cells may potentially overcome the toxic side-effects of the antimetabolite 5-fluorouracil when used in the treatment of cancers, whilst still maintaining its therapeutic effect by preventing any action of the malignant cell to repair DNA synthesis. Human serum albumin microspheres (1-5μm diameter) incorporating 1,5% by weight of 5-fluorouracil were prepared by thermal denaturation at 100°C. The release behaviour of the sustained drug delivery system and the organ distribution of the antimetabolite were evaluated in vitro and in vivo for potential application in the treatment of ovarian cancer. Microspheres (50 mg) with entrapped 5-fluorouracil was added to 10^4 He La cells cultured in Eagles Minimum Essential Medium and the leakage rate of drug determined. Although the initial hourly leakage rate was 0,287 μg 5-fluorouracil, a maximum concentration of 26,688 μg was found at 48 hours. Cell division of the cells were halted before one cell cycle as demonstrated by the absence of micronuclei and double nuclei. Under mild anaesthesia 50 mg 5-fluorouracil-loaded microspheres were injected intraperitoneally into adult female Wirstar rats. The rats were then sacrificed at 2 hours. After exsanguination and collection of urine from the bladder, the major organs were removed. All specimens were freeze-dried and 5-fluorouracil levels determined on each. Highest concentration of 5-fluorouracil was found in the urine (60%), followed by 7,6% in liver, 5,9% in bone, 3,2% in spleen, 3,2% in kidney, 2,6% in faeces, 2,4% in blood, 1,9% in small intestine, 1,8% in ovary, 1,5% in brain, 1,5% in heart, 1,3% in visceral muscle, 0,9% in large intestine, 0,8% in lung, 0,7% in pancreas. The remaining 4,7% was found to be present in body fat, skeletal muscle and glandular tissues. After 30 days, traces of 5-fluorouracil (0,006%) was still detectable in the peripheral blood. Blood chemistry and haematology indicated typical systemic toxic effects of the drug e.g. bone marrow suppression and liver involvement, reaching a maximum by Day 12 after injection. A total return to normal however was observed at Day 24. These data suggest that 5-fluorouracil-loaded albumin microspheres may be beneficial in reducing the severe side-effects of this antimetabolite, whilst still maintaining therapeutic levels to cause tumour cell death.

MICROENCAPSULATION OF BOVINE CHROMAFFIN CELLS: TRANSPLANTATION IN THE RAT MODEL OF PARKINSON'S DISEASE

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Dopamine replacement therapy has been suggested as a possible treatment for Parkinson's Disease. Transplantation of encapsulated heterologous dopamine-producing cells within a permselective membrane may prevent immunological rejection while allowing the diffusion of nutrients into the capsules and the diffusion of dopamine and waste products out. The aim of this investigation was to test the viability of microencapsulated bovine chromaffin cells in vitro and to assess the efficacy of transplanted cells in alleviating motor deficits in the rat model for Parkinson's Disease. In vitro studies compared the level of catecholamines secreted upon depolarization with high potassium saline from free cells versus cells encapsulated in alginate poly-L-lysine alginate microcapsules. In vivo testing compared the effects of sham (empty capsules), free cells and encapsulated cells implanted into the caudate nucleus of rats with unilateral 6-hydroxydopamine lesions of the substantia nigra. Results of the perifusion studies showed comparable levels of catecholamine release from both free and encapsulated cells. High-potassium evoked release of cells cultured for 4 days showed catecholamines released per 1 x 10^4 encapsulated cells were approximately 4700 ng for noradrenaline, 5000 ng for adrenaline and 350 ng for dopamine. Unilateral lesioning of the substantia nigra in rats produced a circling behavior under apomorphine (APO) challenge. APO challenge for 12 weeks following transplantation showed an average of 20% ± 3% reduction in the number of rotations in the sham group, 43% ± 5% reduction in the free cell group; preliminary results show a 65% reduction in the encapsulated cell group. The results demonstrate that encapsulated bovine chromaffin cells retain their ability to secrete catecholamines, that the membrane allows the passage of catecholamines out of the capsule and that intrastriatal transplantation of encapsulated cells leads to an amelioration of symptoms in this rodent model of parkinsonism.

DRUG LOADING AND RELEASE FROM MONOSIZE POLY(ETHYLCYANOACRYLATE) MICROSPHERES

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Monosize, biodegradable polyethylcyanoacrylate (PECA) microspheres in the size range of 0.5-2.5 μm by a phase inversion (dispersion) polymerization. In vitro degradation behaviour of the PECA microspheres was observed, and the effects of pH, temperature and initial concentration of microspheres on degradation were obtained. SEM micrographs showed that PECA microspheres were degraded mainly by surface erosion. Degradation rate increased with an increase in pH and temperature while decreased by an increase in the initial microsphere concentration. A model drug, i.e., 2,4-dinitro-phenylhydrazine was loaded within the PECA microspheres during polymerization. DNPH release from the PECA microspheres with different drug contents was investigated in the media with different pH. Drug loading capacity was increased with the initial concentration of the drug, however, drug loading ratio reduced. The highest drug loading ratio was 56%. The results indicated that, the drug release rate increased by increasing the medium pH and was controlled by degradation of the matrix not the diffusion of the drug. The microspheres were degraded in about 8 hours at physiological pH of 7.4, therefore released their content in this period.

POLY(PHE-LYS) MODIFICATION OF POLYSTYRENE (PS) SURFACES FOR ENZYME IMMOBILIZATION

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In this study, we aimed to modify Polystyrene (PS) surfaces by the polymer of two aminoacids, phenylalanine and lysine, so called poly(phe-lys) to enhance the amino group content of the surfaces for enzyme immobilization. Non-porous PS microspheres (100-300 μm) were prepared by suspension polymerization. Amino group concentrations on poly(phe-lys) treated surfaces were assayed by binding radiolabelled (I-^{14}C) acetic anhydride to the surface, followed by scintillation counter. The surfaces of the plain and modified PS beads were characterized by FTIR spectrophotometer. The amino groups on the Poly(phe-lys) treated PS microspheres were activated by gluteraldehyde. In this part of the study, we exploited a series of enzymes, such as glucose oxidase and invertase which are mainly used in biosensors and food industry. The extent of enzyme binding were investigated by radiolabelling of enzymes with ^{99m}Tc-pertechnetate just before coupling procedure. The K_m values of free and immobilized enzymes were compared at the same experimental conditions specified to each enzyme seperately. We noticed that, for non-porous polystyrene surfaces, the enzyme binding capacity of these surfaces were extended about 20-25 fold than some other type modification techniques given in literature.

ANTIBODY LEAKAGE FROM IMMUNOADSORBENTS : DEPENDENCE ON THE SUPPORT, ACTIVATION PROCEDURE AND ELUTION CONDITIONS

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One side-effect of using immunoaffinity chromatography for the selective extraction of biomolecules or the cleansing of biological fluids, is the leakage of antibodies into the purified media. In the present study, immunoadsorbents (IAs) designed for the selective removal of low density lipoproteins (LDL) by extracorporeal circulation from blood plasma of patients affected by familial hypercholesterolemia, were prepared by covalent coupling of goat antiapolipoprotein B polyclonal antibodies on 4 different supports (Sepharose CL-4B, Sepharose 6 Fast Flow, Sphérodex and Fractogel), previously activated by a variety (cyanogen bromide, divinyl sulphone, tresyl chloride and trichloro-s-triazine) of chemical reagents. Antibody leakage was investigated in relation to the gel type and the nature of its preliminary activation. It turns out that at low pH, the release of antibodies is higher than that observed at neutral pH, especially with the dextran-coated silica gel (Sphérodex). Moreover, the highly cross-linked agarose gel (Sepharose 6 Fast Flow) leads to immunoadsorbents exhibiting both a high yield of coupling, a good adsorption capacity and an excellent stability.

A NEW BIOMATERIAL --- POLYCAPROLACTONE-POLYETHER BLOCK COPOLYMER

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A series of Polycaprolactone-poly (ethylene glycol) block colymers (PCE) with various compositions were synthesized. The blood compatibility of PCE copolymer depends on the polyether content. When the PCE composition was CL/EG=80/20 (mol%), the sample shows the best blood compatibility (the lowest cellular absorbance and the longest blood coagulation time). It is better than that the blood compatibility of polylactide. The biodegradability of PCE copolymer in vitro and in vivo was characterized by hydrolysis reaction with or without enzyme (lipase), and implanted in the back of rats. Results show that the degradation rates of PCE are that: (1) in presence of lipase is faster than in absence of lipase; (2) in vivo is faster than in vitro; (3) in higher PEG content is faster than in lower PEG content. It is revealed that the biodegradability of PCE can be controlled by adjusting the composition of the copolymer. The drug release property of PCE in vitro was identified by using 5-Fluoro-uracil (5-Fu) as a model drug at pH 7.4 and 37℃ . It was found that following with changing composition of PCE the period of half of 5-Fu released (T_{50}) changed from several hours to several months. The 5-Fu release rate increased with increasing both content and molecular weight of the PEG component. It means that the drug release rate of the PCE copolymer can also be controlled by adjusting the composition of the copolymer. Due to the PCE copolymer has a good blood compatibility,improved biodegradability and controllable drug release property, the PCE copolymer is a potential polymer material to be used in artificial kidney, crinical surgery and drug delivery system.

REF: Wang & Qiu, Polymers For Advanced Technologies, 1993,4,363.

SIMULTANEOUS FILTRATION AND ADSORPTION FOR THE REMOVAL OF ENDOTOXINS UTILIZING MICROSPHERES IN A RECIRCULATION SYSTEM

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A new system for a simultaneous filtration and adsorption therapy using an extracorporeal blood purification system was developed. It is based on microspheres which circulate in a secundary circuit. The microspheres are cationically (polyethyleneimine) modified cellulose particles having a size of 3 - 10 µm which adsorb lipopolysaccharides and lipid A (E.Coli 055:B5 and E.Coli F-583, respectively) to a high amount. Falkenhagen et al: Polymers in Medicine and Surgery, 1993, 212. The new system is based on the use of a membrane plasma separator which enables the convection of endotoxins. The plasma filtrate is recirculated by a centrifugal pump (4-8 L/min). Due to the high speed of the recirculating filtrate a very efficient filtrate-flux is established at the inlet of the filter. Hence, the filtrate is being backfiltrated at the outlet of the membrane plasma separator. The secondary circuit contains the microspheres in order to remove endotoxins. The first *in vitro* results clearly demonstrated that the new method of simultaneous filtration/adsorption is able to remove endotoxins from the primary circuit very efficiently. Aqueous solutions but also plasma and blood were investigated. One of the main advantages is its continuous use for several days without stopping the blood flow in the primary circuit. Depending on its efficiency, the adsorbent suspension has to be replaced. In conclusion - a newly developed blood purification system for simultaneous filtration/adsorption based on a high speed recirculation of microspheres was demonstrated to be very efficient to remove endotoxins from aqueous solutions and plasma/blood. The system seems to be an optimal solution for a continuous clinical use in patients suffering from endotoxin shock.

INNOVATION STRATEGIES IN BIOCOMPATIBLE MATERIALS SCIENCE AND ENGINEERING

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Research strategies in engineering innovative biomaterials and parts made from biomaterials mainly take the following into account: a) *non metallics*: (M. D.-dependent) development of metal free load transmitting implants, b)*vital/avital composites*: (M. D.-dependent) development of scaffolds incorporating cells or tissues and respecting minimal lesion surgical application and c) *consumer biomaterials*: development of biomaterials for personal application by the patient (M. D. -independent). Ad a): Metals are not x-ray transparent, they may cause difficulties in diagnostic procedures, i.e. in CT or NMR scans, and incompatible ions or particles (wear) may be released. Polymer based composites with carbon fibers have the potential to replace metals. However careful investigation of long-term behaviour of the fiber-matrix interface, creep behaviour and studies on tissue ingrowth into surfaces is necessary. Ad b): lack of organ and tissue donors will further strengthen research leading to organ and tissue substitutes with incorporated living cells. Biomaterials research providing carriers can profit from micro- and nanotechnology regarding functional surfaces and micromechanical devices. Ad c): Current health care costs may lead to diagnostic and therapeutic use of biomaterials and biocompatible devices independent from application by a medical doctor or may be used in a screening phase before medical consultancy. Regarding these possible scenarios carbon fiber reinforced osteosynthesis devices are presented, non degradable ceramic cell carriers are described and an outlook to consumer biomaterials applications is made.

OBSERVATION OF POLYMERS AND BIOLOGICAL MOLECULES ON SURFACES BY SCANNING TUNNELING MICROSCOPY

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Recently we have constructed a scanning tunneling microscope (STM) and investigated several macromolecules (both natural and synthetic) on highly oriented pyrolithic graphite and mica surfaces. In this paper, we are presenting our experience on STM images. We looked the images of DNA molecules taken from both healthy and cancerogeneous cells, and attempted also to observe interaction of calcuim ions with DNA molecules. We are studying on development of enzyme electrodes in which glucose oxidase and albumin are used in a crosslinked form. In order to understand the effects of crosslinking on the molecular conformations of biological molecules, we took the STM images of these macromolecules and their crosslinked forms. We prepared copolymer micelles from polylactic acid and polyethylene glycol as potential drug carriers. We obtained the STM images of these copolymers, their micelles and also their drug (adriamycin) loaded micelle forms. We studied also micelles of different commercially available emulsifiers used in biological applications (e.g., polyethylene oxide/polypropylene oxide, Tween series, dodecyl benzene sulfonate).

ART. CELLS, BLOOD SUBS., AND IMMOB. BIOTECH, 22(5), A75-A177 (1994)

ABSTRACTS ON

BLOOD SUBSTITUTES

STUDIES ON THE PROCESSING OF THE INITIATION METHIONINE FROM A RECOMBINANT HEMOGLOBIN

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Somatogen has produced a recombinant hemoglobin molecule for use as a blood substitute. This hemoglobin, designated rHb1.1, is expressed in *E. coli* and retains the initiation methionines on the diα and ß N-termini (Looker et al (1992)). Hirel et al. (1989) showed that the extent of Met removal varies according to the identity of the amino acid adjacent to the Met residue. We determined the extent of Met processing from variants of rHb1.1 by the MAP enzyme, in which amino acids of differing side-chain length were inserted adjacent to the initiation Met residue by site-directed mutagenesis. These mutant proteins were analyzed for the extent of Met cleavage by N-terminal sequencing of the isolated diα and ß chains. The functional characteristics of the mutant hemoglobins were determined and compared to those of the parental rHb1.1 protein. The data show that a serine residue positioned adjacent to the initiation methionine produces the greatest extent of Met cleavage, with little change in the functional properties of the hemoglobin. Recombinant hemoglobins with amino terminal serines on the α and ß chains may have some advantages in structure-function studies over the methionine-containing forms.

REF: Hirel et al., Proc. Natl. Acad. Sci. USA 86:8247(1989)

CHARACTERIZATION OF RECOMBINANT HEMOGLOBIN rHb1.1

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Recombinant hemoglobin (rHb1.1) is a modified hemoglobin incorporating both the Presbyterian mutation (B108Asn-->Lys) and a fusion of the two alpha chains by insertion of a glycine between the C-terminal arginine 141 of one alpha chain and the N-terminal valine of the second alpha chain. The protein is composed of three chains: two 146 residue beta chains and one 283 residue fused dialpha chain. The amino termini of the dialpha and beta globins have methionine residues that replace the wild type valine. Using peptide mapping, electrospray mass spectrometry, and RP-HPLC two minor modifications to the dialpha globin were observed; minor modifications to the beta globin were also identified at two sites. Data from size exclusion chromatography indicated the presence of a small quantity of a high molecular weight multimer that is composed of two rHb1.1 molecules joined via a disulfide bond. Additionally, we determined that about 1% of the heme pockets contain protoporphyrin IX rather than heme. Amino acid analysis results indicate that the norleucine (Nle) content is below the limit of detection.

PROTEIN SEQUENCE OF RECOMBINANT HEMOGLOBIN CAN TRIGGER METHYLATION OF N-TERMINAL METHIONINE

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V8 protease peptide mapping of recombinant hemoglobin (rHb1.1) revealed two peptides that are not well resolved by RP-HPLC. Following analysis of the two peptides by sequence determination and electrospray mass spectrometry, we concluded that the N-terminal residue of one of the V8 peptides is modified. We assigned the modification to the N terminus of the dialpha globin as n-methylmethionine. N-methylmethionine has been reported at the N-termini of the ribosomal protein L16 and the bacterial chemotaxis protein CheZ. There is a significant sequence similarity between these two proteins and the dialpha globin of rHb1.1. Several mutants of the dialpha globin were prepared. We have shown that the sequence of the dialpha globin serves as a signal for N-terminal methylation. Mutation of the dialpha globin destroyed the signal, leaving the N-terminal methionine free of modification.

HEMOGLOBIN MODIFIED BY CROSS-LINKING

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Modification of hemoglobin (Hb) is required for the efficacy and safety as blood substitute. Intramolecular and intermolecular cross-linking are the two modification methods that have been investigated most extensively. In our laboratory we first evaluated the intramolecular cross-link of the ß-chains with 2-Nor-2-formylpyridoxal 5'-phosphate (NFPLP). It improves the oxygen affinity, prolongs the vascular retention and prevents kidney damage. By elimination of leakage of Hb through the kidneys, accumulation in the tubuli and increase of enzym leakage from tubular cells was prevented as shown in rat models on exchange transfusion and kidney function. By extrapolation a half life of 8 hours in man can be expected. Further modification by intermolecular cross-linking or polymerization with glutaraldehyde was evaluated in order to achieve a retention time of at least 24 hours. Polymerization of HbNFPLP to polyHbNFPLP resulted in a mixture of polymers of different size. We determined the optimal degree of polymerization with respect to vascular retention time, oncotic activity, viscosity and oxygen affinity. Depending on the degree of polymerization we found a 5- to 7- fold increase in vascular retention compared to native Hb. The change in oxygen affinity was found to be independent of the polymer size. A limiting factor for polymerization is the increase in viscosity. Polymerization to an average molecular mass of 200 kD was considered optimal: a vascular half-life of 15 hours in rats, an iso-oncotic concentration of 9 g Hb/100 ml and a viscosity of 1.2 cp, equal to plasma viscosity. Recently we developed a method for direct polymerization of hemoglobin to polyHb. This product has a number of advantages when compared to polyHbNFPLP: 1) a shorter process time, 2) the relatively expensive NFPLP can be omitted and 3) a higher recovery with respect to the source material hemoglobin. All the abovementioned criteria appeared to be identical to those of polyHbNFPLP. We conclude that polyHb is the most suitable modified Hb to meet the requirements of a red cell substitute to be used as a resuscative fluid under emergency conditions.

RESUSCITATION OF HEMORRHAGE WITH HEMOGLOBIN AND "REPERFUSION INJURY"

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Hypovolemic states are characterized by inadequate tissue perfusion; when this state is reversed, the reintroduction of oxygen in excess may lead to the generation of oxyradicals and presumably cause "reperfusion injury" in susceptible tissues (e.g. kidney, gut, liver, etc.). When hemoglobin in solution is used to reverse such states, generation of oxyradicals may be enhanced by catalytic means. The possibilities were investigated in anesthetized dogs prepared for hemodynamic monitoring, that "reperfusion injury" would occur after resuscitation of hemorrhage with blood, and that such injury would be exacerbated by catalytically-generated oxyradicals when hemoglobin in solution is used for resuscitation. Sodium salicylate was infused to provide a substrate to be oxidized by oxyradicals; the oxidation products generated after extraction of plasma samples with diethyl-ether were measured by HPLC at intervals. Removal of 35 ml/Kg of blood resulted in an acute fall in arterial blood pressure to 39±3/27±3 mmHg, due to a proportional fall in the stroke volume. Reinfusion of the shed blood after 45 minutes of hypovolemia was followed by restoration of the blood pressure and temporary overshoot of the stroke volume. The oxidation products of salicylate, at their peak, rose 3- to 4-fold, indicating the generation and quenching of excess oxidants. Renal function was impaired, in spite of recovery of renal blood flow in both cortex and medulla. Resuscitation with stromafree hemoglobin solution was followed by unsustained recovery of hemodynamics and gross hemoglobinuria accompanied by impaired renal function, in spite of improved of renal blood flow. Salicylate oxidation-products were also increased indicating the generation of oxyradicals. These findings suggest that an effect analogous to "reperfusion injury" occurred under these conditions that may require further investigation. [Supported by Defence and Civil Institute of Environmental Medicine of Canada.]

AN ACUTE ANIMAL EXPERIMENTAL MODEL TO TEST PHYSIOLOGICAL EFFECTS RELEVANT TO HEMOGLOBIN-BASED RED CELL-SUBSTITUTES

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An acute experimental model has been developed to permit testing the physiological responses of several organ systems to hemoglobin-based red cell-substitutes in dogs and is also applicable to swine. The preparation can be used in models of defined hemorrhage (either to defined volume or to a defined blood pressure), or in exchange transfusions; in the latter case coincidental hemodynamic perturbations can be minimized to describe responses to the test substance alone. After induction and tracheal intubation, the animals are ventilated at fixed rate and volume using N_2O/O_2/Penthrane. Through cut-downs catheters are placed in the femoral arteries and veins and a Swan Ganz catheter is inserted through the right external jugular vein and positioned appropriately. The left chest is opened and a catheter is inserted in the left atrium. The bladder is catheterized; the right femoral arterial catheter is advanced and, through a small abdominal incision, is manipulated into the right renal artery. This preparation permits repeated blood sampling for the estimation of various enzyme activities that may be released from damaged cells. Central hemodynamic variables can be determined and microsphere injections (either radionuclide- or dye-labelled) are used to measure organ blood flows from which regional and organ vascular resistances and hindrances can be derived. Renal functional parameters can be determined using simultaneous arterial and renal venous samples and timed urine collections. By these means, such parameters can be determined as electrolyte excretion and clearance, the clearance and true extraction of creatinine and assay of urinary N-acetyl-galactosidase (N.A.G.) activity as a guide to the integrity of the luminal border of the tubular epithelial cells that may be the target of hemoglobin filtered through the glomerulus. This model has been used to determine the response in bled dogs (35 ml/Kg) to the infusion of SFHS. [Supported by Defence and Civil Institute of Environmental Medicine of Canada.]

EVALUATION OF IMMUNOGENICITY OF MODIFIED HUMAN HEMOGLOBIN SOLUTIONS

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We are currently developing a blood substitute based on human hemoglobin that is modified by intra- and intermolecular crosslinking with glutaraldehyde. In the present study we investigated the possibility that neoantigens are introduced by the modification. To investigate the potential immunogenicity of the polymerized hemoglobin (polyHb) we analyzed antibody responses of rabbits after intramuscular adminstration with Freunds complete adjuvant. In view of the species differences we also tested rabbit hemoglobin that was modified in the same way as human polyHb. Thereafter, we studied the antibody response after weekly intravenous infusion of relatively large doses of polyHb (100 mg/kg), to evaluate whether an immune response is likely to occur when modified hemoglobin is used as blood substitute. The occurrence of an antibody response was tested for using an enzyme immunoassay (ELISA). To find out whether antibodies were directed against neo-epitopes on human polyHb we used competitive ELISA in which native hemoglobin competes with modified hemoglobin that is immobilized on the solid phase. The results showed that polymerized hemoglobin carries neo-epitopes that may activate the immune system in special conditions, but are unlikely to do so when polyHb is used as blood substitute.

OXYGEN-BINDING ALBUMINS: A NOVEL APPROACH TO BLOOD SUBSTITUTES

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Serum albumin is the second most abundant protein in human blood. A 70kg human being has about 750 grams of hemoglobin and 375 grams of serum albumin. The terminal half-life for exogenously administered albumin is approximately two weeks. To date, blood substitute research has focussed either on stroma-free hemoglobin or perfluorocarbon-based products. Clinical trials have been carried out on products derived from both approaches. Each has been shown to have its own particular problem. Stroma-free hemoglobin, for example, is vasoactive in a possibly deleterious way; and perfluorocarbons deliver oxygen by simple rather than facilitated diffusion. A well known function of serum albumin is its binding of various serum-borne compounds, including hemin. We have been testing the hypothesis that a serum albumin containing a transition metal-based oxygen-binding site can be prepared. To this end, we have made metal-based "picket fence porphyrins" bound to human serum albumin. These "oxygen binding albumins" reversibly bind oxygen, although their O_2-binding capacity decreases gradually due to oxidation of the heme iron. We (Kilbourn, et al., 1994) have also shown that one of these oxygen-binding albumins has no significant effect on phenylephrine-evoked contraction of rat aortic rings treated with endotoxin. Physicochemical properties of oxygen-binding albumins will be discussed. This work was supported by Apex Bioscience, Inc.

REF: Kilbourn, R.G., G. Joly, B. Cashon, J. DeAngelo, & J. Bonaventura, Biochem. Biophys. Res. Comm., 199:155-162. 1994.

FUNCTIONAL PROPERTIES OF HEMOGLOBINS INTRAMOLECULARLY CROSSLINKED WITH TRICARBOXYLIC AND DICARBOXYLIC ACIDS.

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The esterification of carboxyl groups with 3,5-dibromosalicylate groups produce a very mild activation of the acyl residues. These esters of di- and tri-carboxylic are very little reactive with either α- or ϵ-amino groups. This is why they produce only intramolecular crosslinks of hemoglobin, reacting with lysines, which are shielded from the solvent, so to have their pK substantially lowered. Intramolecular crosslinks are produced either between the α99 or β82 lysines respectively. The increasing length of the crosslink between the β subunits from 4 to 14 carbon atoms progressively decreases the oxygen affinity of hemoglobin. Preliminary data suggest that the same may be true for the crosslinks between α subunits. The triester of benzene-tricarboxylic acid produces very homogeneous β-β crosslinked hemoglobins, with low oxygen affinity.

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Hemoglobin Pilot Scale Production and Characterization of Liposome Encapsulated Hemoglobin as an Artificial Red Blood Cell

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This study is concerned with the development of both hemoglobin (Hb) production process and an effective and safe red blood cell substitute-liposome encapsulated Hb (LEH). A pilot scale production unit for the preparation of stroma free Hb (SFH) from outdated human red blood cells according to "red blood cell ghosts" procedure is described. Products of the facility should be of adequate quality to address most of the research toxicity and efficacy issues facing further development of Hb-based red cell substitutes. The fully automated (data acquisition / control) IBM PC supported system, consists of haemolysis unit, which contains outdated human red blood cells between two 800 nm membranes, cross-flow filtration system with four filter packages of 450 nm, 220 nm, 300 kD and 100 kD and ion exchange chromatography column. Hb concentration is performed on 10 kD membrane inside cross-flow system. According to this procedure, stroma and pyrogen-free Hb is prepared for development of Hb-based red cell substitutes and evaluation of their in-vitro and in-vivo (rats) properties. LEH red blood cell substitute is developed, based on modified film hydration and reverse-phase evaporation methods. The membrane lipids, used to encapsulate Hb solution included phosphatidylcholine (PC, Soy or Egg), cholesterol, sphingomyelin, α-tocopherol and distearoyl phosphatidylethanolamine (PEG-DSPE), in molar ratios for liposomal membrane formulation of large unilamelar vesicles (200 nm) that results in prolonged circulation time.

A STARCH-HEMOGLOBIN RESUSCITATIVE COMPOUND

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A resuscitative compound in freeze-dried form has been synthesized between a modified starch and a tetramerically stabilized hemoglobin. In order to complex the hemoglobin, the starch has been prepared in mono-, di-, tri- and tetra-aldehyde moieties. The hemoglobin was stabilized with low molecular weight diacids. Electrophoretic densitometric patterns indicate compound formation. The resulting polymers were characterized with respect to the number average molecular weight (osmotic pressure), second virial coefficient, intrinsic viscosity, oxygen transport (biotonometry), Hill constant, P_{50} and Bohr effect.

The in vitro evaluation indicates that these compounds are effective hemodiluents, offer protection to the red cell membrane and do not cause erythrocyte aggregation.

The final product is a cost-effictive acellular resuscitative compound which can be stored in freeze-dried form at room temperature for extended periods of time. This artificial blood substitutes can be reconstituted upon the addition of water.

ACTIVATED NEUTROPHILS AS A TEST SYSTEM FOR EVALUATION OF CORPUSCULAR INTRAVENOUS PREPARATIONS.

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Neutrophils are the first cells of an organism that contact and interact with foreign corpuscular micro particles. The interaction turns the cells into reactive state, which is characterized by an activation of the main interrelate functions of neutrophils including the production of oxygen active forms. A luminol enhancement chemiluminescence (CL) micro-assay was developed for an evaluation of the interaction of activated peritoneal neutrophils with perfluorochemical emulsions. The cells were obtained from the peritoneal cavity of mice 5 hrs. after zymosan injection at a dose of 0.5 mg. The cells were incubated in 0.2 ml cuvettes containing 0.1 mM luminol. CL was monitored by means of luminometer LX-12 that allows to make a simultaneous analysis of 10 samples. Time resolution was approx. 1 min. Addition of perfluorochemical emulsions to neutrophils results in an increase of CL as if it takes place under interaction neutrophils with opsonized zymozan. Analysis of CL features shows that cells "feel" emulsion particle diameter, quality and concentration of emulsions. It is suggested the technique may be useful for a creation of the cell-invisible fluorocarbon blood substitutes and other intravenous preparations.

HEMODILUTION WITH MODIFIED FLUID GELATIN ACCELERATES THE PASSAGE OF PLASMA (NOT RED BLOOD CELL) THROUGH CEREBRAL MICROVESSELS IN RATS

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Passage of red blood cells (RBCs) through cerebral microvessels is much more rapid than that of plasma in awake rats. Isovolemic hemodilution with modified fluid gelatin lowers the total circulatory RBC mass as well as blood viscosity, thereby, may alter the passage time of RBC as well as plasma through cerebral microvessels. This study was designed to clarify this question. Adult Wistar-Kyoto (WKY) rats, aged 28-40 weeks, were used. The animals were divided into the hemodilution and the control groups. Isovolemic hemodilution, which lowed the systemic hematocrit (sHct) to the level of 30-35%, was achieved by rapidly replacing blood with the same volume of 3% modified fluid gelatin. Local cerebral blood flow (LCBF), and microvascular RBC (Vr), and plasma (Vp) volumes in 14 brain structures were measured using ^{14}C-IAP, 55Fe-labeled RBCs, and ^{14}C-inulin, respectively. The cerebral microvascular blood volume (Vb)=Vr+Vp, the cerebral microvascular hematocrit (mHct)=Vr/Vb. The mean transit times of blood (Tb), RBC (Tr), and plasma (Tp) through cerebral microvessels were as follows: Tb=Vb/LCBF, Tr=Tb x (mHct/sHct), and Tp=Tb x [(1-mHct)/(1-sHct)]. The results showed that in the control group, the mHct in the 14 structures ranged 26-40% with a mean of 35%, which was 69% of the mean sHct (51%). Tb, Tr and Tp were 0.63-1.82sec (mean=0.94sec), 0.46-1.17sec (mean=0.64sec). and 0.8-2.5sec (mean=1.26sec), respectively. In the hemodilution group, the mean mHct was 28%, which was 88% of the mean sHct (32%). LCBFs of the 14 structures were about 60% higher than those of the control animals. Vbs of the 14 areas were similar to those of the control group. Mean Tb was 0.62sec (66% of the control), Tr was 0.5sec (84% of the control), and Tp was 0.66sec (52% of the control).These findings indicate that isovolemic hemodilution with modified fluid gelatin results in a marked shortening of the time for the passage of plasma (not RBC) through cerebral microvessels, that is , accelerates the plasma flow velocity in cerebral microvessels.

THERAPEUTIC EFFECT OF HEMODILUTION WITH MODIFIED FLUID GELATIN ON RATS WITH IMPENDING HEAT STROKE

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Heat stroke is one of the medical emergencies during Summer in Taiwan. The patients reveal severe functional failure of the brain, kidney and liver. Therefore, we have hypothesized that these phenomena may be related to the prolonged organ ischemia. Furthermore, high blood viscosity and increased blood cell counts noted in patients with heat stroke may exacerbate the organ ischemia. The purposes of this study were to investigate: 1) whether organ ischemia developed before the onset of heat stroke; 2) whether hemodilution could reverse organ ischemia in animals with impending heat stroke. Adult male Sprague-Dawley rats weighing ~250g were used. The animals were divided into 3 groups. The first group was the room temperature (RT) group, in which the animals were placed in room temperature (26^{o}C) for 2 hours and 10 minutes. The 2nd group was the heat stroke (HS) group, in which animals were put in high ambient temperature (42.5^{o}C) for 2 hours, then were moved to room temperature for 10 minutes. The 3rd group was the hemodilution (HD) group, in which the animals were prepared as those in the 2nd group except that hemodilution was performed immediately after the animals were moved out from the high ambient temperature. Hemodilution was achieved by rapidly replacing blood (4-6ml) with modified fluid gelatin (8-10ml) in one minute. With this procedure, systemic hematocrit was lowered from ~50% to ~30%. Blood flow was measured in liver, kidney and 14 various brain structures by using the ^{14}C-iodoantipyrine method at 2 hours & 10 minutes exposure to the various ambient temperature. The results showed that in the RT group, the mean body temperature was 37^{o}C, renal blood flow was 526$\pm$68ml/100g/min, hepatic blood flow was 50$\pm$9 ml/100g/min, local cerebral blood flows (LCBFs) in 14 brain structures ranged from 115$\pm$17 ml/100g/min in the medulla oblongata to 283$\pm$56 ml/100g/min in the frontal cortex. In the HS group, body temperature was 41$\pm$0.1^{o}C, renal and hepatic blood flows were 475$\pm$23, and 44$\pm$3 ml/100g/min, respectively. LCBFs in all 14 brain areas were markedly decreased by 50% as compared to those of the RT group. In the HD group, hemodilution effectively lowered body temperature from 41$\pm$0.3^{o}C to 38$\pm$0.3^{o}C and increased renal and hepatic blood flows to the level of 646$\pm$69 and 98$\pm$23ml/100/min, respectively, LCBFs in all 14 brain areas were also reversed to the same levels of the RT group. In conclusion, cerebral (not hepatic or renal) ischemia develops before the onset of heat stroke. Hemodilution with modified fluid gelatin reverses th cerebral ischemia in animals with impending heat stroke.

THE MORPHOLOGY OF INTERACTION BETWEEN UN-MODIFIED HEMOGLOBIN AND ERYTHROCYTES

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The stroma-free hemoglobin (Hb) prepared from human and pig bloods were characterized by analytical methods including light scattering, electrophoresis (SDS-PAGE and IEF), and HPLC. The results show no significant difference between human and pig hemoglobins. Also, the compatibility characteristics of unmodified hemoglobin (Hb) in human blood were tested by factor activation, coagulation cascade, fibrinlytic process and by observation of morphology changes of interaction of unmodified Hb with human erythrocytes under microscope. The results indicate that no significant interaction may observed for up to 50% volume change of blood with hemoglobin solution (at 7.5g/dl). Thus, hemoglobin with limited volume ratio of Hb solution might be a fair attempt of temporary treatment with acute cerebral microcirculation blocking.

POLYETHYLENE GLYCOL MODIFIED HEMOGLOBIN IS ABLE TO SUSTAIN LIFE IN THE CONSCIOUS PORCINE EXTREME EXCHANGE TRANSFUSION MODEL.

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Eight Yorkshire cross pigs (20-30 kg) were implanted with Data Sciences (St. Paul, MO) blood pressure probes two weeks prior to the study. Fully conscious pigs were randomized into two groups: a control group to receive Dextran-70, and a test group to receive 6% polyethylene glycol modified bovine hemoglobin (PEG-Hb). Pigs underwent exchange transfusion (ET) by repeated 10% exchanges until either a hematocrit less than 10% was achieved, or physical signs indicated a close to lethal endpoint. Total blood volume was calculated at 70 ml/kg. Blood was exchanged at a rate of 0.7 ml/kg/min. Prior to ET, at every 10% exchange, and daily after the ET, pigs were monitored for the following: blood pressure, heart rate, respiratory rate, hematocrit, RBC count, Hb concentration and profile, blood gases and physical examination. PEG-Hb showed no effect on PaO_2, PvO_2, $PaCO_2$, pH, arterial Hb saturation of O_2, respiratory rate or heart rate in these fully conscious adolescent swine. The results demonstrated that PEG-Hb was able to sustain life in pigs with hematocrit levels below 10%, indicating successful transport of oxygen to body tissues.

THE EFFECTS OF CROSSLINKING HEMOGLOBIN WITH SUPEROXIDE DISMUTASE AND CATALASE ON FREE RADICAL SCAVENGING ACTIVITY

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Free radical processes may mediate the adverse physiologcal effects observed with hemoglobin-based red cell substitutes. To eliminate this possibility we cross-linked hemoglobin, superoxide dismutase, and catalase (PolyHb-SOD-catalase). Our studies show this product scavenges oxygen-derived free radicals and reduces methemoglobin formation and iron release under oxidative conditions. It was also observed that during storage at 4°C and 20°C, methemoglobin formation was reduced in PolyHb-SOD-catalase compared to PolyHb solutions. In the xanthine/xanthine oxidase system, the initial rate of cytochrome c reduction was 2.13 ± 0.26 nmoles cyt. c/min for PolyHb alone. PolyHb-SOD-catalase reduced this to 0.56 ± 0.08 nmoles cyt. c/min because of its ability to eliminate superoxide ($O_2^{\cdot}$). Addition of PolyHb to 200 μM of hydrogen peroxide (H_2O_2), changed the H_2O_2 level slightly to 192 ± 0.4 μM. Addition of PolyHb-SOD-catalase, on the other hand, lower the level to 41 ± 0.3 μM. Oxidative challenge with H_2O_2 resulted in minimal changes in the absorbance spectra of PolyHb-SOD-catalase. With PolyHb, there were spectral changes reflecting the formation of methemoglobin and heme degradation. Furthermore, the amount of iron released, after incubation with 250 μM H_2O_2, was 6.8 ± 1.8 μg/dl for PolyHb-SOD-catalase and 76.6 ± 1.0 μg/dl for PolyHb. By scavenging oxygen-derived free radicals, PolyHb-SOD-catalase may reduce in vivo free radical reactions with hemoglobin components and prevent other in vivo oxidative processes.

NITRIC OXIDE INTERACTIONS WITH HEMOGLOBIN IN VIVO

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Hemoglobin and red blood cell lysates have been found to act as vasoconstrictors, both in vitro and in vivo. The mechanism by which Hb functions in this manner may involve NO scavenging. The NO related vasoactivity of cell-free hemoglobin in blood substitutes is an undesirable side-effect which is a critical issue in determining their safety. On the other hand, the great avidity of hemoglobin for NO and its ability to convert it to higher oxides of nitrogen can be translated into a number of new applications for cell-free hemoglobin solutions. We have been studying hemoglobins in vitro and in vivo, and have found that cell-free hemoglobin reverses the endotoxin-mediated hyporesponsivity of rat aortic rings to alpha-adrenergic agents. Cell-free hemoglobin also abrogated the profound drop in blood pressure seen in a septic shock model in dogs. In a rat solid tumor model, we have found that nitric oxide scavenging by cell free hemoglobin reduces tumor blood flow and leads to decreased tumor perfusion. This effect is achieved in tumors with only a mild and transient systemic pressor effect. We believe that the use of hemoglobin as a nitric oxide scavenger has numerous potential clinical applications. This will provide useful clinical knowledge which will contribute to the scientific platform needed to overcome current problems relating to undesirable side-effects encountered in the use of hemoglobin-based blood substitutes.

RESTORATION OF TUMOR OXYGENATION AFTER CYTOTOXIC THERAPY BY A PERFLUBRON EMULSION/CARBOGEN BREATHING

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Female, Fisher 344 rats bearing 13762 mammary carcinoma implanted subcutaneously in a hind limb were treated with standard therapeutic single doses of antitumor treatments of several types including: 1) antitumor alkylating agents (cisplatin, cyclophosphamide); 2) natural products (adriamycin, taxol and etoposide); 3) antimetabolites (5-fluorouracil); 4) hypoxic cell selective cytotoxic agents (mitomycin C, SR-4233) as well as 5) fractionated radiation therapy (3 Gray daily for 5 days). The oxygen levels in the tumors were measured in the absence of treatment and 24 hrs. after treatment using an Eppendorf pO_2 histograph. Fifty- to sixty- points were measured per tumor and 8-10 tumors comprised each group. The tumors were more hypoxic post treatment with every anticancer drug or radiation. The percent of pO_2 readings $\leq$ 5 mmHg in the untreated tumors was 49% and in the treated tumors ranged from 85% (x-rays) to 59% (etoposide). Administration of the perflubron emulsion (8 ml/kg) and carbogen breathing (95% O_2/5% CO_2) increased the oxygenation of the tumors such that the percent of pO_2 readings $\leq$ 5 mmHg was 32% in the untreated controls and ranged from 27% (x-rays) to 56% (adria) in the treated tumors. Hypoxia protects malignant cells from the cytotoxicity of many drugs and radiation. These results indicate that administration of a perflubron emulsion/carbogen can increase the oxygen content of tumors when hypoxia is the result of cytotoxic therapy. [Supported by NIH grant P01-CA19589 and a grant from Alliance Pharmaceutical Corp., San Diego, CA.]

WHOLE BODY PROTECTION DURING ULTRAPROFOUND HYPOTHERMIC PROCEDURES USING A NEW INTRACELLULAR-TYPE AQUEOUS BLOOD SUBSTITUTE SOLUTION.

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Hypothermia is a very useful adjunct technique for selected neurosurgical & cardiovascular procedures. But as in any major surgery that employs cardiopulmonary bypass (CPB), the demand for multiple postoperative transfusions of blood and blood products is considerable. The blood substitute could also serve as an in situ universal hypothermic preservation solution thus protecting the brain and systemic organs. Eleven dogs were blood substituted with an "intracellular-type" aqueous blood substitute specifically designed to protect the brain and visceral organs during an extended period of at least 3 hr of controlled ultraprofound hypothermia <10°C, (Cryomedical Sciences, Inc., Rockville, MD) and three with an "extracellular-type" control solution. Both solutions are without any specific oxygen carrying molecules. The anesthetized animals were cannulated for extracorporeal pump oxygenation. Dogs were exsanguinated (hematocrit <1%) during cooling and volume replaced with the oxygenated cold solution (PO2 >500 mmHG). After 3 hr of cardiac arrest and continuous perfusion, rewarming began. The solution was drained and the animals were autotransfused, weaned from the pump, decannulated, and recovered. In the experimental group, eight animals continued to have survived long term (current range = 12-100 weeks) without experiencing any detectable neurological deficits. In the control group, two dogs survived after extensive cardiac resuscitation efforts. A wide range of biochemical & hematological parameters were monitored on days 1, 2 and 3 and weeks 1, 2 and 3 postoperatively. Only inconsequential and transient elevations in enzymes were observed in the experimental group as compared with greater elevations in the control group. Immediate postoperative values (mean ± SE) for SGOT were: 89 ± 19 vs. 734 ± 540, $p<.01$; for SGPT: 46 ± 4 vs. 98 ± 52, $p<.05$; for LDH: 114 ± 11 vs. 490 ± 209, $p<.001$; and on day one for CK-BB: 108 ± 22 vs. 617 ± 154, $p<.01$. These studies clearly show the significance of "intracellular-type" blood substitute in protecting the brain in hypothermic procedures with cardiac arrest.

DEVELOPMENT AND VALIDATION OF A COMPUTER MODEL FOR EVALUATION OF EFFICACY OF PERFLUOROCHEMICAL EMULSIONS

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It is generally accepted that venous blood oxygen tension (PO_2) reflects (but does not measure) PO_2 of the tissue from which it is issuing and that mixed venous PO_2 (PvO_2) is an acceptable indication of the oxygenation status of the whole body. It would be logical therefore to use PvO_2 as an indication for the need for blood transfusion during surgical procedures and in the trauma situation. A computer program has been developed to predict PvO_2 under a variety of clinical and experimental conditions. Inputs are grouped into those determining the position of the oxyhemoglobin dissociation curve and those determining oxygen transport and delivery. If Hb concentration, oxygen consumption (VO_2), arterial and mixed venous blood gas are entered, O_2 transport and VO_2 variables for both red cell contained Hb and for the plasma phase are obtained together with PvO_2. By inputing details of plasma phase oxygen carriers, such as hemoglobin solutions or perfluorochemical emulsions, their influence on permitted blood loss and relationship to PvO_2 and Hb concentrations can be determined, allowing the calculation of allowable blood loss before transfusion becomes necessary. With this information, techniques can be designed for maximizing the benefit of oxygen carriers in surgical autologous blood strategies. The accuracy of prediction of PvO_2 has been validated using animal and human data. The program indicates that a small dose of Oxygent (emulsion of perflubron [perfluorooctyl bromide], Alliance Pharmaceutical Corp.) can allow the loss of up to 2/3 of blood volume while maintaining PvO_2 at or above predosing levels. These predictions have been validated in an anesthetized dog model employing hemodilution.

DO THE DISSOCIATION OF THE DEX-BTC-HB AND ITS OXIDATION INFLUENCE THE P50 IN VIVO IN GUINEA PIG?

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Dextran-benzene-tetracarboxylate-hemoglobin (dex-BTC-Hb) is a potential blood substitute whose oxygen affinity (P50 = 20-22 torr) and vascular remanence (half time = 9,5±0,5hrs) are both satisfactory. In previous studies (1), we showed the fast but limited oxidation of Hb in plasma (35±5% maximum in 24hrs) which indicated that most of the circulating Hb was suited to carry oxygen. However, in the present study, we cannot positively state that Hb remains bound to dextran-BTC polymers and maintains a satisfactory P50.
In this work, we investigated dex-BTC-Hb dissociation, hemoglobin oxidation and its O2 affinity in plasma.
We performed a 50% blood mass exchange in guinea pigs with a 70±5g/l dex-BTC-Hb solution. After taking regular blood samples during the 24hrs subsequent to the exchange, the dissociation state of plasmatic dex-BTC-Hb was determined with HPLC (column TSK G3000 SW on Varian LC 5020) in phosphate buffer pH 7,2 (flow rate 1ml/min, 403 nm). The P50 was determined by Hemox Analyzer (TCS Medical Products, USA) in a Bis Tris Buffer pH 7,4 at 37°C and the metHb rate was determined according to Kaplan's method.
The results demonstrated the very slow dissociation of dex-BTC-Hb with more than 70% of circulating hemoglobin remaining bound to dex-BTC during the first 24hrs following the transfusional exchange. However, at the same time, we noticed a drop in P50 which seemed to correlate with the formation of methemoglobin.

(1) B. Faivre *et al* Methemoglobin formation after administration of hemoglobin conjugated to carboxylate dextran in guinea pigs. Attemps to prevent the oxidation of hemoglobin. Biomat. Art. Cells, Immob. Biotech., 1993, in press.

ASSESSMENT OF DEXTRAN 10 -BENZENE-TETRACARBOXYLATE - HEMOGLOBIN, AN OXYGEN CARRIER, USING GUINEA PIG ISOLATED BOWEL MODEL.

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In order to of assess dextran-benzene-tetracarboxylate hemoglobin as an oxygen carrier, we studied histological changes in the intestinal loop. The intestinal tissue being very sensitive to hypoxia in the anesthetized guinea pig, an innervated loop was vascularly perfused with an open-flow during one hour at zero hematocrit (1). To estimate the capacity of hemoglobin solution to oxygenate this tissue, we observed the mechanical and histological changes in the organ and the arterio-venous difference in PO2, oxyhemoglobin, deoxyhemoglobin and we compared them with human albumin, Tyrode and non-modified hemoglobin solutions.

The PO2 arterio-venous differences were 51.9±7.1torr (m±SEM) for Tyrode solution (n=3), 40.2±6.4 torr for albumin solution (n=3), 113.7 ± 6.5 torr for non-modified hemoglobin (n=5) and 123.1 ± 7.9 torr for dex-BTC-Hb (n=5). Compared to albumin and Tyrode, hemoglobin solutions released more oxygen into tissues and the oxygen quantity increased with time. The desaturation of dex-BTC-Hb was significantly increased ($p<0.05$) compared to non-modified hemoglobin. The structure of jejunal villi, when perfused with a hemoglobin solution, remained almost normal and the loop was still active. Nevertheless, non-modified hemoglobin leaked from the vessels to the lumen and caused an oedema and a rupture of the overlapping epithelium at the tip of the villi. With dex-BTC-Hb, such histological modifications were less significant. With albumin and Tyrode, the loop was completely inert and all villi were totally necrosed.

We have demonstrated that dextran-benzene-tetracarboxylate hemoglobin had the ability to maintain the tissue alive thanks to its good capacity to release oxygen and its satisfactory vascular persistence. A Hb-dex-BTC solution can answer oxyphoric needs in tissues and could be used to preserve organs.

(1)B.Faivre, P. Labrude, C. Vigneron. Assessment of histocompatibility of different hemoglobin solutions using mesenteric perfusion on the small bowel of Wistar male rat. Biomat., Art. Cells & Immob. Biotech. 1991, 19 (3), 521-537.

REACTION OF NITRIC OXIDE WITH HEMOGLOBIN

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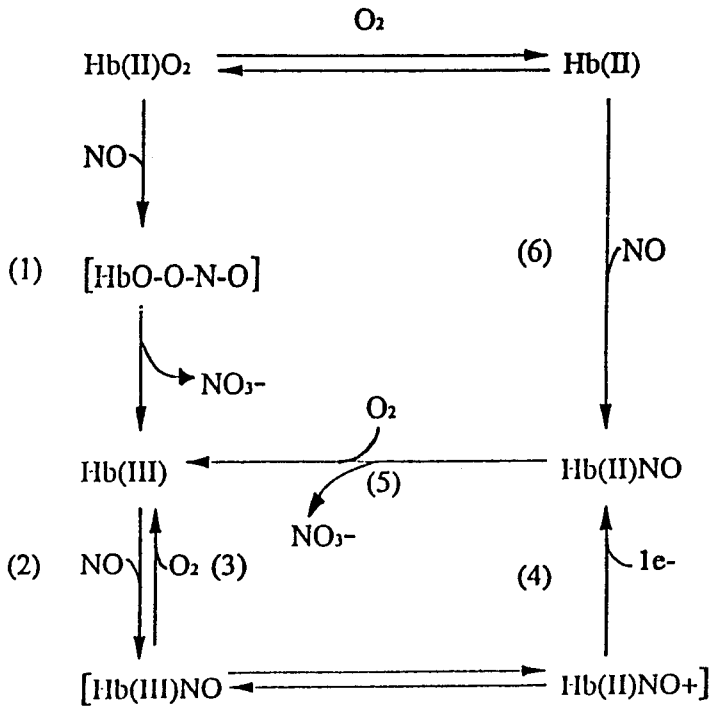
The reaction of hemoglobin with nitric oxide has been investigated by recording absorbtion spectra of human hemoglobin in time, in the presence of different ligands. The results are summarized in the figure. They suggest that the combination of the various derivatives of hemoglobin with NO would converge to the formation of methemoglobin [Hb(III)], establishing a redox cycle which depletes the nitric oxide being produced.



MODULATION OF NITRIC OXIDE AFFINITY BY AMINO ACID RESIDUES IN THE POCKET OF THE B-CHAINS.

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Vasoactivity represents a major concern for the *in vivo* use of cell free hemoglobin. It is believed to result from the interaction of hemoglobin with the endothelium-derived relaxing factor (EDRF). There is strong evidence that EDRF is in fact nitric oxide (NO). The high affinity of NO for hemoglobin is due to its extremely low dissociation reaction. An increase in NO dissociation will result in an decreased affinity for NO. We have observed that in a recombinant hemoglobin β67Val→Thr the rate of NO release was increased 10 fold. A similar increase was measured for fetal hemoglobin where Ser replaces Ala 70 and a Leu replace Phe 71 of human β-chains. These data indicate that the affinity for NO can be affected by amino acid substitutions in the heme pocket. Under the same experimental conditions used for the kinetic experiments the oxygen affinity of HbA and HbF were 7.2 and 4.5 mmHg respectively indicating that HbA had a lower oxygen affinity than HbF. Conversely, the kinetic experiments indicate that the kinetic of NO release was one order of magnitude higher for HbF than HbA, suggesting a different response of NO and O_2 binding parameters to amino acid substitutions in the heme pocket of the β-chains.

CRYSTALLOGRAPHIC AND STRUCTURAL ANALYSIS STUDIES OF RECOMBINANT AND CHEMICALLY MODIFIED HEMOGLOBINS DESIGNED TO AID DEVELOPMENT OF ARTIFICIAL HEMOGLOBIN-BASED OXYGEN CARRIERS

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Crystallographic studies of liganded and unliganded forms of recombinant and chemically-crosslinked forms of human hemoglobin have been undertaken to assist in the development of artificial hemoglobin-based oxygen carriers. A number of new structures of mutant and cross-linked hemoglobins have been completed. These structures have been determined using data from crystals that are isomorphous with previously crystallized hemoglobins for which structures are available. The crystal structure solution of each variant involves the collection of high resolution x-ray data using an electronic area detector followed by difference Fourier interpretation, alteration of the starting model followed by restrained least-squares refinement. The structures will be described and compared with natural and other hemoglobins with similar properties. These variants include, among others, β(Val1Met + ΔHis2), β(Val67Thr) and sebacyl hemoglobin. (The sebacyl hemoglobin is prepared by crosslinking the two βLys82's with bis-(3,5-dibromsalyicyl)sebacate.)

TRANSFUSIONS USING RED BLOOD CELLS WITH MODIFICATION OF SURFACE ANTIGENS.

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The ability to maintain a constant supply of universal-blood type or blood group O red cells, cells which can be transfused to recipients of any blood group, has spurred our interest in the biochemical manipulation of red cell surface carbohydrate structures. Blood groups A, B and O have carbohydrate-defined specificities. Groups A and B are formed in vivo by the attachment of one additional specific sugar residue to the already-assembled blood group O carbohydrate chain; in the case of A antigen, this terminal residue is an α-linked N-acetyl-D-galactosamine and for B, it is an α-linked D-galactose. It should be possible, by using appropriate glycosidases, to reverse this process in vitro, thereby creating blood group O. Our studies on converting group A red cells to group O are still at an in vitro stage because of the structural complexity of the A_1 antigens, some of which contain dual A antigenic sites, one terminal and one internal to the carbohydrate chain. Our efforts in converting group B erythrocytes to group O have progressed much further. We have shown that cells can be enzymatically treated under conditions which maintain RBC integrity and viability in vitro and have demonstrated the normal life span, safety and efficacy of these cells in vivo when they are transfused in volumes up to two full units (400 mL RBC) to group A and O healthy volunteers. These clinical studies are continuing with the administration of three units of enzymatically converted group B cells to A and O recipients. Supported by ONR grants # N00014-93-1-0466 and N00014-94-I-0189 with finds provided by NMRDC

NITROSYL HEMOGLOBIN FORMATION IN-VIVO AFTER INTRAVENOUS ADMINISTRATION OF A HEMOGLOBIN BASED OXYGEN CARRIER IN ENDOTOXEMIC RATS

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Interaction of HBOCs with nitric oxide (NO) of endothelium or macrophage origin has been implicated in the obsevered vasoconstriction of some HBOC infusion. Definitive evidence supporting this interaction, in-vivo, has not been reported. We report here a confirmed in-vivo formation of nitrosyl Hb (HbNO), a product of Hb and NO reaction, in endotoxemic rats following intravenous administration of a HBOC. Sprague-Dawley rats were given an intravenous injection of lipopolysaccharide (LPS, 100mg/Eg's). Five hours later a 1/3 blood volume (2% of body weight) of HBOC (10gHb/dl) was infused. Venous blood samples were taken before LPS, prior to HBOC infusion, and 1 hour post HBOC administration. Erythrocyte and plasma HbNO levels were determined using electron paramagnetic spin resonance (EPR) spectroscopy. Erythrocyte samples taken 5 hours post-LPS exhibited the characteristic hyperfine signal of HbNO at g=2.021, 2.011, and 2.001, indicating intra-erythrocytic Hb binding of NO diffused across the red cell membrane. No HbNO signals were detected in either the 5 hours post-LPS plasma or baseline blood samples. At 1 hour post-HBOC, approximately equal size HbNO signals were detected in plasma and erythrocyte samples. This is direct evidence of NO reaction with infused Hb. In conclusion, HBOC interacts in-vivo with NO directly in a model with increased NO. Whether this effect is present at endogenous levels of NO requires exploration.

OXYGENATION OF SKIN TISSUE AND DRUG DELIVERY BY POSPHOLIPID- ENCAPSULATED FLUOROCARBONS

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Besides the use of fluorocarbon emulsions as blood substitutes, specially designed phospholipid - carriers bearing fluorocarbons, are able to penetrate the skin surface. Various fluorocarbons were coated with an uneven number of phospholipid lamellae by homogenization in an aqueous medium. The particle diameter were in the range of 100- 240 nm. The lipids used were highly enriched phosphatidylcholin fractions of soy lecithin. The lamellar structur of particlecoating was confirmed by cryo-transmission electron micrographs, NMR- investigations and electrical measurements.

The vesicle- like structure of aggregates favours their topical application . Channeling molecular oxygen from the outside to skin tissue, cells will be oxygenated enhancing skin metabolism and cell respiration. Additionally, encapsulated fluorocarbons may function as vehicles to carry diagnostic agents, pharmaceuticals and other lipophilic principles. Drugs and vitamins dissolved in fluorocarbon, are transported via the stratum corneum into lower dermatic tissue. The penetration rate is controlled by the size of aggregates. While the phospholipids were taken up by cell membranes in soft fusion, drugs can act at a definite place or generally in body after being taken up by the blood stream.

The penetration rate additionally depends on PFC lipophilicity as it was found for saturation of liver microsomal membranes. The critical solution temperature of fluorocarbons in n- hexan (CST) qualitatively describes the solubility of PFCs in lipids. Varying this property, e.g. by mixing fluorocarbons of different CSTs, the residence time can be controlled.

As a diagnostic agent a nonreactive persistent fluorocarbon radical was encapsulated in phospholipids by the sonication method. After topical administration at isolated fresh mouse skin, the penetration was observed using an EPR- tomographic method. The increasing oxygen tension was measured at skin depending on time with the oxygen- sensitive Clark - electrode. After treatment of human skin with the PFC- carrier, the pO_2- pressure at skin surface has nearly doubled.

REVIEW OF MODIFIED HEMOGLOBIN RESEARCH AT LETTERMAN: ATTEMPTS TO DELINEATE THE TOXICITY OF CELL-FREE TETRAMERIC HEMOGLOBIN

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In the final two years, June 1991 to June 1993, of the Letterman Army Institute of Research a variety of cell, tissue, organ, and animal systems were used to explore the toxicities of model hemoglobin (Hb) solutions produced in the sterile Hb production facility. Human mononuclear cells release TNFα and Il-8 when exposed to chromatographically purified human Hb (HbA_0). Mixed cultures of fetal mouse neurons and glial cells exhibit neuronal death with exposure to HbA_0 in a dose and time dependent manner while the glial cells are not injured. Isolated perfused rabbit hearts were used to explore the reversibility of coronary vasoconstriction after Hb and cyanomet-Hb administration, and deferoxamine was shown to partially protect that reversibility. In rabbits HbA_0 and human Hb cross-linked with *bis*(3,5-dibromosalicyl) fumarate (ααHb) caused hypertension and pulmonary arteritis. In swine, HbA_0 and ααHb caused systemic and pulmonary hypertension and a doubling of the vascular resistance that was equivalent to that seen with inhibition of nitric oxide synthesis. Elevations of creatine kinase and lactic dehydrogenase activity were observed after Hbs, but not after blockade of nitric oxide synthesis. Acute renal failure seen after administration HbA_0, did not appear after ααHb. Infusion of cyanomet-ααHb did not cause the increased vascular resistance seen after ααHb. The infusion of l-arginine or nitroglycerine with ααHb did not prevent the increased vascular resistance and decreased cardiac output or allow the increased oxygen carrying capacity provided by Hb in the plasma from translating into improved oxygen delivery or improved oxygen consumption.

MULTILINKING REACTIONS BETWEEN HEMOGLOBIN TETRAMERS IN SOLUTION AND THE CRYSTAL

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Increasing the molecular mass of a crosslinked hemoglobin may increase the plasma retention time and the amount of oxygen delivered at the same osmotic pressure. To test the hypothesis, an octamer of hemoglobin can be made by crosslinking between two tetramers. For solution reactions, longer, hydrophilic tetra-aspirin reagents can be used to connect two tetramers. Tetralinkers and crosslinkers of different lengths can be used to give preferences for inter- or intra-tetramer reactions. In the crystalline state, the distances between neighboring tetramers are desirably short and fixed. Using computer simulation of the deoxyhemoglobin crystal, the amino groups of Lys 7 and Lys 11 on one tetramer in a unit cell and Lysß66 on another tetramer were selected to be specific sites for crosslinking. These sites are on the pathway of a channel between the tetramers through which small molecules can diffuse in the crystal. Tri(3,5-dibromosalicyl) tricarballylate was designed by computer graphics to fit this site and made as a trilinker for these specific sites. The stability and oxygen binding properties of the crosslinked hemoglobins will be described. (Supported in part by a grant from the Research Corporation.)

EFFECTS OF HALOTHANE AND ISOFLURANE ON DIASPIRIN CROSSLINKED HEMOGLOBIN™-INDUCED CONTRACTIONS OF PORCINE PULMONARY VEINS

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Diaspirin crosslinked hemoglobin (DCLHb™) is a resuscitative fluid presently undergoing clinical trials. Administration of DCLHb is associated with an elevation of mean arterial pressure *in vivo* and contraction of isolated blood vessels *in vitro*[1]. The mechanisms for the vascular actions are unknown but may be due to inhibition of nitric oxide (NO) release from vascular endothelium. Halothane has been reported to inhibit NO induced relaxation[2]. We examined the effect of anesthetics on DCLHb induced contraction of blood vessels. Porcine pulmonary veins were excised, cut into rings and placed in organ chambers filled with 25 ml Krebs-Ringer solution (37°C). Following equilibration at their optimal length the rings were exposed to increasing concentrations of serotonin(10^{-8}M-10^{-5}M). Endothelial activity was confirmed by relaxation greater than 80% with ACh (10^{-6} M). DCLHb (1.5×10^{-8}M to 6×10^{-7}M) contracted porcine pulmonary veins (1.04 ± 0.17g to 3.45 ± 0.22g), and halothane (0.5% and 1%) significantly inhibited these DCLHb induced contractions in a dose related maner (-41.6 ± 8.1% and -73.3 ± 8.2%, respectively). At equimolar concentrations, isoflurane had less inhibitory activity than halothane. The relative inhibitory effect of these volatile anesthetics is consistent with their inhibitory actions on other heme containing proteins[3]. These results suggest that halogenated anesthetics may interact with the heme iron of DCLHb and inhibit its binding with NO.

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EFFECT OF PERFLUOROCHEMICAL EMULSION (FLUOSOL®) ON SHEAR INDUCED BLOOD TRAUMA.

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The major problems of development and improvement of heart-assist devices and prosthetic heart valves is the reduction of blood cell damage. The prolonged contact and impact between blood and foreign surfaces, extremely high shear forces and other abnormal hydrodynamic circumstances have been shown to cause intensive blood trauma: hemolysis, activation of platelets, and undesirable changes of mechanical properties of erythrocytes. Hemolysis, in turn, can drastically increase erythrocyte aggregation at low shear conditions. We investigated a new pharmacological approach to reduce blood trauma and improve rheological properties of blood subjected to mechanical stress. Preliminary experiments (*in-vitro*) with ovine, bovine and human blood showed that the replacement of 10-20% plasma volume with FLUOSOL® (Alpha Therapeutic Corp., Los Angeles, CA), a perfluorochemical that transports oxygen, significantly ($p<0.005$) reduced mechanical fragility of erythrocytes. The Mechanical Fragility Index decreased from 0.737 ± 0.090 to 0.490 ± 0.091. The same replacement of plasma with FLUOSOL® reduced hemolysis (plasma free Hb) by approximately 60% compared to control, during *in vitro* pumping of ovine blood with a centrifugal pump. A 20% replacement of plasma volume with FLUOSOL® remarkably reduced low shear blood viscosity (by two times) and erythrocyte sedimentation rate (by approximately 10 times) in human blood. The decrease of these parameters indicates the reduction of erythrocyte aggregation. Results of this study-in-progress demonstrate the potential feasibility of FLUOSOL® to decrease blood trauma in circulation-assist devices.

SIMULATION OF OXYGEN SATURATION OF HEMOGLOBIN SOLUTION, RBC SUSPENSION AND HEMOSOME BY NEURAL NETWORK SYSTEM

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Hemoglobin-based artificial blood substitutes as oxygen carrier is advantageous over current plasma expander. In this studies, oxygen saturation of hemoglobin solution, red blood cell suspension and artificial blood substitute under various conditions were measured by yeast-consuming-oxygen experiments instead of spectrophotometer. The former method provides direct measurement of oxygen dissociation without destroying the lipid membrane of RBCs and hemosomes for the reason of light scattering. The empirical results were assigned into training feedforward back-propagation neural network system in order to simulate the oxygen saturation model modulated by those factors such as pH, temperature, $[Cl^-]$, [2,3-DPG], P_{O_2} and P_{CO_2}. The interaction of these factors were considered simultaneously in this neural network model with comparison to other models with combination of independent empirical equations proposed by Heck, Kelman and so on. Consequently, this neural network model is able to simulate accurately the oxygen saturation of Hb solution, RBC suspension and hemosomes. Moreover, it is also applicable to predict oxygen saturation of hemosome in human blood for future clinical trial.

PRODUCTION PFC SUBMICRON EMULSIONS AND LIPOSOMES WITH HIGH PRESSURE HOMOGENIZER "DONOR-1"

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It is well known, that production of PFC emulsions has many specifical features. We have built up high pressure homogenizer "Donor-1" specially adapted for production from small (50 ml) to large (50 l) quantities of PFC emulsions or liposomes. Homogenizer has an original construction. He is supplied by two identical homogenizing devices, working interdependently one from other. Herewith, each homogenizing device can smoothly change its capacity just in work process from 0 to 500 ml/min. Electrodriver produces work pressure in wide limits from 0 to 200 MPa independently for each homogenizing devices. Each homogenizing devices can be cooling with liquid Homogenizing devices can easily take to pieces for washing and can to be sterilized by autoclaving. There is a large choice of homogenizing valves. All this possibilities allows to get exclusive combinations of different conditions for preparation of PFC emulsions and liposomes. Homogenizer "Donor-1" provides production of submicron PFC emulsions with average particle diameter 50-70 nm with narrow particle distribution. We investigated dependence of average particle diameter and particle distribution from such technological factors as pressure, temperature, PFC and surfactant concentration and of configuration of homogenizing valves.

NEW PFC SECOND GENERATION EMULSIONS ON THE BASE OF PERFTORDECALIN (PFD) OR PERFTOR-OCTILBROMID (PFOB) WITH ADDITION PERFTOR-METHILCYCLOHEXILPIPERIDIN (PFMCP)

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We propose the emulsions consisting of fast eliminated PFC in quantity 40-50 v/v% with addition a small quantity 1-10 v/v% more slowly eliminated PFC and stabilized with phospholipids in quantity 2-6 w/v%. The fast eliminated PFD ($C_{10}F_{18}$) or PFOB ($C_8F_{17}Br$), which transfer the main quantity of oxygen, has a half life times 7 and 4 days, while PFMCP ($C_{12}F_{23}N$) has 65 days. Although PFMCP himself too transfer the oxygen, the main role of PFMCP is to stabilize the emulsion and to reduce toxic. Emulsions were prepared with homogenizer "Donor-1" (Russia). Particle diameters were measured with analyzer "Coulter N4" (USA). The grey rabbits with mass 2,5-3,0 kg were used. We estimated that PFD emulsion has for rabbits LD_{50}=10-15 ml/kg and doze 20 ml/kg causes 100% death after 5-7 days. But PFD/PFMCP emulsion takes off the toxic effect of PFD. Rabbit survival after injection of PFD/PFMCP(9:1) emulsions in doze 20 ml/kg was 100%. We observed reduction of lung edema. PFD/PFMCP and PFOB/PFMCP emulsions has high stability. For PFD/PFMCP(9:1) emulsion particle average diameter after preparation was 240 nm and after about 3 years of storage (+4^{o}C) was 265 nm.

The Results of a Phase I Clinical Trial of a 40 v/v% Emulsion of HM351 (Oxyfluor™) in Healthy Human Volunteers.

Robert J. Kaufman

A 40 v/v% emulsion of the novel perfluorocarbon HM351 was previously reported to be stable to terminal sterilization at 121°C and to have good shelf stability properties. HM351 was also found to be free from pulmonary hyperinflation effects found with many other perfluorocarbons (PFCs) and to have an acceptably short tissue residence time.

Oxyfluor™ was tested in a Phase I clinical trial in healthy human volunteers at three doses: 0.25, 0.5 and 1.0 ccPFC/kg (0.62, 1.25 and 2.5 cc Emulsion/kg). There were six controls and six treatment subjects at each dose level. Control groups received a comparable volume of 0.9% saline. The subjects and the medical staff were blinded throughout the study. A 0.5 mL test dose was administered to each subject to test for clinical signs of complement activation. The subjects were given the full dose and monitored for vital signs, cardiac status, pulmonary function, hematology, clinical chemistry, coagulation, urinalysis and complement activation during infusion and at 4 hours, 1, 2, 4, 7, 14, 21 and 30 days post infusion. Pharmacokinetics of the PFC were also measured by gas chromatography.

There were no cardiac or respiratory abnormalities observed in these subjects: Electrocardiogram and 2-D echocardiography were all normal. Pulmonary function tests (FEV1 and FVC) and respiration rate were normal at all doses and timepoints. Coagulation and clinical chemistry were within normal ranges at all doses and tmepoints. There was no evidence of complement activation. The observed side effects included a mild and transient thrombocytopenia at the highest dose and dose responsive flu-like symptoms seen previously with other PFC emulsions. The flu-like symptoms started about 4 hours post infusion were resolved by 24 hours post infusion.

The flu-like symptoms appear to be related to release of cytokines and arachadonic acid metabolites and can be reversed in animals by premedication with indomethacin or dexamethasone.

The intravenous half-life of Oxyfluor™ was dose responsive. At 1 ccPFC/kg, the half-life was 2.3 hours.

FACTORS AFFECTING O_2 DELIVERY BY PERFLUOROCHEMICAL EMULSIONS DURING ACUTE NORMOVOLEMIC HEMODILUTION

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It is generally recognized that the greatest utilization of transfused blood is during surgical procedures, and that the use of autologous blood carries less risk and is preferred over the use of allogeneic blood. Presently, autologous techniques include predonation, acute normovolemic hemodilution (ANH), and salvage. "Blood substitutes" can augment these autologous strategies by supporting tissue oxygenation during blood loss in surgery, thereby preserving the autologous blood until later in surgery or once hemostasis has been achieved. Oxygen delivery to tissues by perfluorochemical (PFC) emulsions is quite different from that of red cells or acellular hemoglobin (Hb) solutions. All PFCs physically dissolve O_2 in direct proportion to the partial pressure of oxygen. Hb has a fixed capacity to bind O_2; therefore O_2 delivery is proportional to the dose and requires O_2 dissociation from the heme and diffusion of the O_2 across the red cell membrane and into the plasma prior to transfer to the tissues. Comparing the efficacy of a PFC emulsion to blood or Hb requires a consideration of the O_2 extraction. Normally, only about 25% of the O_2 transported by the Hb is extracted and consumed compared to >90% O_2 extraction from the PFC emulsion. In addition to increases in the arterial PO_2, cardiac output (CO) is a critical parameter in determining the contribution of PFC-dissolved O_2 to oxygen consumption (VO_2). The normal physiological response to ANH is an increase in CO due to lower blood viscosity, less peripheral resistance, and increased venous return. A PFC-based oxygen carrier such as *Oxygent™*; (perflubron-based emulsion, Alliance Pharmaceutical Corp.) that does not affect the blood pressure or interfere with the normal increase in CO will offer the greatest contribution to VO_2 when used in conjunction with ANH. Thus, O_2 delivery by PFC emulsion is based on its linear O_2 solubility, the contribution of virtually all of its O_2 to tissue VO_2, and the additional benefit derived from the increased CO during ANH. Moreover, the dissolved O_2 carried by a PFC emulsion is present in the plasma compartment (i.e., it does not have to diffuse across the red blood cell membrane), thereby enhancing the gradient for diffusion of O_2 to the tissues.

HEMOGLOBIN MEDIATED VASOACTIVITY IN ISOLATED VASCULAR RINGS

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To define hemoglobin-based oxygen carrier (HBOC) vasoactivity, a preliminary study with isolated vascular ring preparations was performed. Transeversal ring segments of the pulmonary artery and aorta were obtained from male SD rats. The vessel rings were mounted in a tissue bath containing Krebs buffer (pH=7.4, 37C) and instrumented to isometrically record changes in circumferential tension. Norepinephrine (NE) 15-70uM significantly increased vascular tension (26-180% over pretreatment values, N=8, P<0.01) which was partially reversible by 50-400 uM sodium nitroprusside (SNP) or 10-50 uM glyceryl trinitrite (GTN). Human stroma-free Hb (SFH; 6-8gHb/dl) at 4-15 uM significantly increased isometric tension (29.4±16.8%, N=12, P<0.001) of vessels predilated with SNP. Methemoglobin 0.5-2uM did not produce significant effect. The vasoconstriction of SFH was reversible by nitro vasodilators (e.g., glyceryl trinitrite), SNP, and beta receptor agonist isoproteranol. In conclusion, SFH at micromolar concentration caused a substantial vasoconstriction in isolated arterial rings but was reversible with nitro and other vasodilators. The isolated vessel preparation is a sensitive model for testing vasoactivity. In the absence of central nervous system influence and other factors (e.g., circulating blood cells, hormones, etc.), this model allows direct study of Hb effects on vessels.

BIOCONJUGATION OF HOMOGENEOUS CROSS-LINKED HEMOGLOBIN BY CHEMICAL DESIGN

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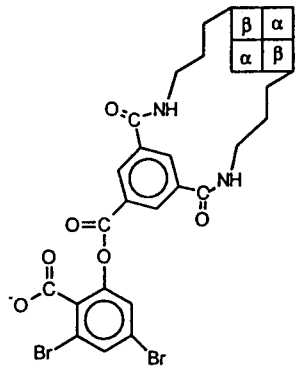
The reaction of trimesoyl tris(3,5-dibromosalicylate) with deoxy-hemoglobin produces almost exclusively a cross-linked product in which the ε-amino groups of both β-Lys-82 side chains are connected to one another while the third (uniquely reactive) group of the reagent remains unmodified:



Reaction of the cross-linked hemoglobin with a series of nucleophilic biomolecules efficiently produces conjugates in which the dibromosalicylate group is replaced. The method is versatile and a number of applications in combining drug delivery with a blood substitute are readily accessible. Properties of the resulting hemoglobin can also be adjusted readily.

TWO TYPES OF TOTALLY SYNTHETIC OXYGEN CARRIER: - LIPOSOME EMBEDDED HEME(L/H) AND LIPID HEME MICROSHERE(LH-M)- .

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Many red blood cell substitutes have been proposed. Most of them are derived from human or bovine hemoglobin and some of them are under preclinical study.

We have produced two types of totally artificial oxygen carrying substances. In both systems, synthetic hemes are used as oxygen carrier. In liposome embedded heme system (L/H),synthetic hemes are embedded in bilayers of liposome as vehicles of hemes. In lipid heme microshere system(LH-M), synthetic hemes are arranged to cover the surface of fat microsheres as vehicles of hemes.

Design, physiochemical characteristics, solution properties, oxygen binding capacity in vitro of both material will be discussed. Also in vivo oxygen carrying ability of the L/H and LH-M was studied in dogs undergoing hemorrhagic shock by drawing 30 ml/kg of blood. Thity minutes after induction of shock, the same volume of either the L/H or the LH-M solution was intravenously injected. In the L/H group, 30 minutes after the L/H injection, calculated oxygen delivery(DO_2) by L/H was 19 % of total DO_2 and calculated oxygen consumption(VO_2) from the L/H was 24 % of total VO_2. In the LH-M group, 30 minutes after the LH-M injection, calculated DO_2 by the LH-M was 16 % of total DO_2 and calculated VO_2 from the LH-M was 15 % of total VO_2. Thus, both L/H and LH-M have ability as oxygen carrier.

A NEW TOTALLY SYNTHETIC OXYGEN CARRIER: LIPID HEME MICROSHERE(LHM)

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Fat microspheres, clinically used for fat substitutes, are covered with synthetic hemes to produce totally artificial oxygen carrier. In this system the fat microspheres play role as vehicles of synthetic hemes. The diameter of this lipid heme microsphere(LHM) is 0.2μ and its P_{50} is 40 hPa. Specific gravity, viscosity and osmotic pressure of the LHM can be adjustable similar to that of human blood. Oxygen carrying ability of the LHM is studied in dogs undergoing hemorrhagic shock by drawing 30 ml/kg of blood. Thity minutes after induction of shock, the same volume of the LHM solution was intravenously injected. Oxygen delivery by the dogs' hemoglobin and the LHM are seperately calculated. Also oxygen consumption from the dogs' hemoglobin and the LHM are seperately calculated. Thirty minutes after the LHM injection, calculated oxygen delivery by LHM is 16 per cent of total oxygen delivery and calculated oxygen consumption from the LHM is 15 per cent of total oxygen consumption. Thus, this LHM has ability as an oxygen carrier.

STUDY OF THERAPEUTIC EFFICIENCY OF NEW BLOOD SUBSTITUTES IN EXPERIMENTAL HEMORRHAGIC SHOCK

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Blood substitutes prepared on the basis of dextran and used currently have a number of drawbacks, so the search for new blood substitutes are being made in Russia and abroad. Volekam (Institute of Technology Blood Substitutes, Moscow) and Polyoxidin (Russian Research Institute of Hematology & Transfusiology, S.-Petersburg) are the objects of the present study. Volekam is 6% solution of oxyethyl starch in 0.9% NaCl solution, relative viscosity is 3.0-3.6, pH 5.0-7.0. Average MW of oxyethyl starch is 170 000 D, polydispersity coefficient is 3.0-3.5. Polyoxidin is a 1.5% solution of polyethylene glicol in 0.9% NaCl solution, relative viscosity is 1.5-2.0, pH 5.0-7.6. Average MW of polyethylene glicol is 20 000 D, polydispersity coefficient is 1.00-1.09. In experiments on dogs and rats, which were anesthetized and underwent hemorragic shock, the hemodynamics, oxygen transport and acid-base state were studied. Cardiac function was studied using ultrasound equipment, microcirculation, by the method of vital contact microscopy. It is established that administration of Volekam and Polyoxidin corrects hypovolemia with subsequent restoration of arterial pressure in the aorta and cardiac cavities and increase in the myocard contractility and cardiac output. Comparative analysis has shown that Polyoxidin exerts a more prolonged action than Volekam: the oxygen transport increases to a greater extent and correction of the acid-base balance is more effective with Polyoxidin. In addition, Polyoxidin lowers the blood and plasma viscosity, decreases the blood cells aggregation which promotes the restoration of peripheral circulation. Efficiency of Volekam and Polyoxidin is not inferior to that of well known blood substitute Polyglukin (dextran group) and, in respect of come parametrs, is even superior to that of the latter.

SMALL VOLUME RESUSCITATION OF HYPOVOLEMIA USING HYPERTONIC SALINE-HEMOGLOBIN

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Stroma-free hemoglobin (Hb) solutions treat hemorrhagic shock by increasing oxygen delivery (DO_2) resulting from increased oxygen blood content and modest increases in vascular volume. The useful dose of Hb may be limited to small volumes due to increased peripheral resistance. Hypertonic 7.5% saline-6% dextran (HSD) treats shock by increasing cardiac output (CO) and DO_2 by volume expansion, increased contractility, and reduced peripheral resistance; but resultant hemodilution reduces oxygen blood content. Using published data[1,2] we modeled the effects of 15% Hb, HSD and 7.5% NaCl-15% Hb (HS-Hb) on cardiac output, volume expansion, and DO_2 in hemorrhagic shock. Table shows DO_2 (ml/min) during hemorrhagic shock and at 10 and 30 minutes after 5 ml/kg infusion in simulated prehospital resuscitation, and after simulated hospital resuscitation where CO was normalized in all groups with lactated Ringer's.

	baseline	shock	10 min.	30 min.	hospital
Hb alone	1114	397	440	491	733
HSD	1114	397	841	655	708
HS-Hb	1114	397	897	708	745

Effective prehospital resuscitation of moderate hemorrhage was not achieved with Hb solution because CO was only modestly increased and hematocrit was reduced. Hb solutions for the initial treatment of trauma will require infusion of large volumes, or combining the hyperoncotic Hb with a hyperosmotic crystalloid in order to be effective.

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ON BIOPHYSICS MECHANISMS OF TOXICITY OF FLUOROCARBON EMULSIONS

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Intravenous administration of emulsions of some perfluorochemicals (PFCs) are followed by lung gas-exchange alterations, lung inflation and animal death. A low aggregation stability of toxic perfluorodecalin emulsion into the blood stream was revealed by means of freeze fracture electron microscopy. On the other hand, this emulsion produces pulmonary gas trapping (PGT). It is suggested the PGT phenomenon is based on the different molecular masses of fluorocarbons and alveolar gases (nitrogen, oxygen, carbon dioxide and water vapor). The mass difference of fluorocarbon and alveolar gases provides a 4-fold difference in their diffusion rates. Therefore, lighter alveolar gases will diffuse into alveoli more quickly then fluorocarbon vapor will diffuse out of the alveoli to the environment. This effect results in additional gas pressure into alveoli and, as a consequence of it, PGT and lung inflation. The simple working model of the phenomenon is presented. It is the contact between air and hydrogen separated by porous partition that results in an appearance of the additional pressure in the compartment containing more heavy gas. Theoretical and experimental analysis shows that (1) an absence of emulsion particle aggregation in the blood stream, (2) a low pressure of saturated vapors of the PFC phase and (3) a relatively low velocity of PFC expiration from the organism are essential conditions for the creation of a safe fluorocarbon blood substitute.

STABILITY OF PERFLUOROCARBON EMULSIONS AND THEIR COMPATIBILITY WITH BLOOD SERUM

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The stability of perfluorocarbon (PFC) emulsions and their behavior in a vascular bed depend on a surface layer cohesion of surfactant around the particles.The study of solely a particle size of these dispersed preparations is not enough to judge of their stability and quality.It is essential to evaluate also the integrity of structure of particles and their compatibility with the biological media (blood,plasma,serum),i.e. biocompatibility of PFC emulsions.Approaches to the study of the above said properties of PFC emulsions have been elaborated.The stability and biocompatibility with blood serum of PFC emulsions at different temperatures of storage have been studied.It was shown that the stability and biocompatibility of PFC emulsions of the same composition depend on the method of their preparing.The average diameter of PFC emulsion particles may remain unchanged at the storage,but the biocompatibility may worsen.The particle size of frozen PFC emulsions as a rule does not change,and their biocompatibility with blood serum can remain unchanged or even improve.However, the integrity of particle structure of PFC emulsions of another composition may be destroyed in unfreezing.Study of the above physico-chemical parameters is nesessary to improve the PFC emulsions quality.

INTRAMOLECULAR CROSSLINK OF HUMAN HEMOGLOBIN WITH A LONG CHAIN DICARBOXYLIC ACID BETWEEN THE α SUBUNITS.

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It is well known that short chain dicarboxylic acids (fumaric,succinic) activated by 3,5-dibromo-salicyl groups produce intramolecular crosslinks between either the α99 Lys or between the β82 Lys residues. We recently reported on crosslinks between the β82 lysines using 10,12, and 14 carbon dicarboxylic acids. We found that the incubation mixture of human deoxyhemoglobin with bis-(3,5-dibromosalicyl)sebacate (10 carbons long) contains three main intramolecularly crosslinked molecular species. The major component is a hemoglobin crosslinked between the β82 lysines,as reported elsewhere. The other minor fractions are crosslinked either between the α chains only,or between both the α and β chains. All of these species have low oxygen affinity with P50 above 30 mmHg. Preliminary data suggest that the α subunits are crosslinked between the α99 residues.

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EFFECTS OF HEMODILUTION WITH HEMOSOME ON THE LOCAL CEREBRAL BLOOD FLOW OF CAROTID-OCCLUDED RATS

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Our recent experimental results showed that the lowered local cerebral blood flow (LCBF) of the carotid-occluded rats can be normalized by hemodilution with modified fluid gelatin (geloplasma), however, the forebrain remained hypoxic since geloplasma could not carry oxygen to the ischemic brain. To improve this adverse effect of hemodilution, hemosome, a lecithin encapsulated hemoglobin which has oxygen-carrying ability, was made for using as the hemodilution fluid. Therefore we have hypothesized that the ischemic forebrain of the carotid-occluded rats could be normalized by hemodilution with hemosome. Male Sprague-Dawley rats weighing ~350g were divided into three groups. The fist group was the non-ligation, non-hemodilution group. The second group was the ligation , non-hemodilution group, in which the bilateral carotid arteries were ligated. The third group was the ligation, hemodilution group, in which the carotid-ligated rats were treated with hemodilution by rapidly replacing blood with hemosome. With this procedure, the systemic hematocrit was lowered from ~50% to ~30%. LCBFs were measured in 14 brain structures of the awake rats using the ^{14}C-iodoantipyrine technique. Our results revealed that LCBFs of the carotid-occluded rats decreased by ~50% in all 14 brain structures, which included 8 forebrain and 6 hindbrain structures. These partial ischemic forebrain structures became severe ischemia after hemodilution with hemosome, for example, the LCBFs in the sensorimotor cortex were 283±30, 61±10, and 4±2 ml/100g/min for groups 1, 2, and 3 , respectively. In the hindbrain structures, hemodilution with hemosome could reverse their ischemic condition, for example, LCBFs in the medulla oblongata were 115±17, 83±10, 139±9 ml/100g/min for group 1, 2, and 3, respectively. In conclusion, hemodilution with hemosome fails to reverse the ischemic forebrain of carotid-occluded rats.

VASOACTIVE EFFECTS OF STROMA FREE HEMOGLOBIN.

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We have compared the effects of unmodified SFH and an iso-oncotic solution of albumin on mean arterial blood pressure (MAP) and glomerular filtration rate (GFR) in the rat *in vivo* following induction of hypovolemic hypotension by acute hemorrhage (removal of 20ml blood/kg body weight). Hemorrhage reduced MAP from 114±2 to 63±3mmHg and GFR from 2.8±0.2 to 1.0±0.2ml/min respectively. Subsequent infusion of unmodified SFH increased MAP and GFR to baseline levels (111±3mmHg and 2.7±0.3ml/min respectively). In contrast, albumin infusion increased MAP to 82±2mmHg and GFR to 2.0±0.2ml/min. Thus, the increase in MAP and GFR induced by albumin was substantially less than with SFH. Infusion of Hemosafe ®, a modified (0-raffinose crosslinked) SFH had effects on MAP and GFR that were comparable to that of the unmodified SFH. We next did studies to elucidate the potential role played by NO inactivation in the observed changes in MAP induced by SFH. Rats subjected to hemorrhage received either albumin or unmodified SFH following which the NO synthase inhibitor, L-NAME was administered while continuing the infusion of albumin or SFH. In hypotensive rats treated with albumin, L-NAME increased MAP from 82±4 to 104±3mmHg. In contrast, NO inhibition had no effect on MAP in hemorrhaged rats infused with SFH (111±3 to 112±3mmHg). These studies indicate that NO availability is markedly reduced by SFH, explaining the absence of any hypertensive effect of L-NAME following SFH administration. We have previously reported that SFH results in intrarenal vasoconstriction and reduced GFR in the isolated perfused kidney (IPK)(1). Our data demonstrating an SFH-induced *decrease* in GFR in the IPK and an *increase* in GFR in the hypoperfused kidney following hemorrhage *in vivo* appear contradictory. However, these data are compatible with other studies reported by this laboratory in which NO synthase inhibition (with either L-NAME or L-NMMA) reduced GFR when administered to euvolemic rats and increased GFR in hemorrhaged, hypotensive rats in which the kidney is hypoperfused (2,3). On the basis of these studies we hypothesize that the ultimate effect on renal function of NO synthase inhibition, as well as of NO inactivation by SFH administration, depends upon the prevailing renal perfusion pressure. In conclusion, SFH raises MAP as well as GFR in the hypotensive, hypovolemic rat in part by expanding intravascular volume (via a colloid effect) and in part by peripheral vasoconstriction induced by inactivation of NO. Thus SFH administration has the potential to result in acute beneficial effect on MAP and renal function when administered in states of hypovolemic shock.

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HEMODILUTION WITH MODIFIED FLUID GELATIN NORMALIZES THE PROLONGED PASSAGE TIME OF RED CELLS AND PLASMA THROUGH CEREBRAL MICROVESSELS IN THE PARTIAL ISCHEMIC FOREBRAIN OF RATS

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In awake rats, the mean transit time of red blood cell (RBC) through cerebral microvessels (Tr) is much shorter than that of plasma (Tp). Our recent results also indicate that isovolemic hemodilution lowering the systemic hematocrit (sHct) to the level of 32% accelerates the passage of plasma (not RBC) through cerebral microvessels. However, little is known about the alterations of Tr and Tp in the partial ischemic forebrain; nor about the effects of isovolemic hemodilution on Tr and Tp in the partial ischemic brain. This study was designed to elucidate these questions. Wistar-Kyoto (WKY) rats, aged 30-40 weeks, were divided randomly into three groups. The first group was the ligation-hemodilution group, in which animals were treated with bilateral common carotid artery ligation and, then, isovolemic hemodilution by replacing blood with the same volume of 3% modified fluid gelatin. The second group was the ligation, non-hemodilution group, in which animals were treated with bilateral carotid artery ligation. The third group was the non-ligation, non-hemodilution group. LCBF, and microvascular RBC (Vr) and plasma (Vp) volumes in 14 brain structures were measured using ^{14}C-IAP, 55Fe-labeled RBCs, and ^{14}C-inulin, respectively. These 14 structures were divided into the caudal hindbrain, the rostral hindbrain, the forebrain structures. The cerebral microvascular blood volume (Vb)=Vr+Vp; the cerebral microvascular hematocrit (mHct)=Vr/Vb; mean transit time of blood through cerebral microvessels (Tb) = Vb / LCBF; Tr = Tb x (mHct/sHct), Tp=Tb x [(1-mHct)/(1-sHct)]. Two hours after the carotid artery ligation, LCBFs were decreased by 38% in the forebrain structures, 22% in the rostral hindbrain areas, and 8% in the caudal hindbrain. In contrast, Vbs were increased by 68%, 37% and 16% in the 3 regions, respectively. Tr and Tp were all prolonged (180% for Tr and 154% for Tp) markedly in the forebrain regions, moderately (91% for Tr and 73% for Tp) in the rostral hindbrain, and mildly (60% for Tr and 13% for Tp) in the caudal hindbrain. In the ligation-hemodilution group, Tr and Tp in the forebrain were similar to those of the non-ligation, non-hemodilution group. However, Tr and Tp in the rostral and hind brain structures were much less than those in the non-ligation, non-hemodilution group. In conclusion ligation of the bilateral common carotid arteries in WKY rats causes a partial forebrain ischemia, in which both Tr and Tp are prolonged. These prolongations on Tr and Tp can be normalized by isovolemic hemodilution with modified fluid gelatin.

SYNERGISTIC ENHANCEMENT OF PROTOPLAST-DERIVED COLONY GROWTH BY PERFLUORODECALIN AND PLURONIC F-68

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Division of plant protoplasts is enhanced by culture in medium overlaying oxygenated perfluorodecalin and supplemented with the non-ionic surfactant, Pluronic F-68 (Anthony *et al.*, 1994). To extend these preliminary findings, the subsequent growth of protoplast-derived cell colonies from such systems has been assessed by image analysis. Cell suspension-derived protoplasts of albino *Petunia hybrida* cv. Comanche were cultured for 10 days (2.0×10^5 protoplasts ml^{-1}) at the interface between KM8P medium (2.0 ml) containing 0.01% (w/v) Pluronic F-68, overlaying oxygenated (10 mbar for 15 min) perfluorodecalin (BNFL Fluorochemicals Ltd., U.K.; 6.0 ml) in 30 ml glass bottles. At 10 and 17 days, KM8P medium (0.5 ml) was replaced with an equal volume of KM8 medium (of reduced osmotic pressure) and the total population of cell colonies was transferred (on day 40) to the surface of 10.0 ml of agar-solidified UM medium in 5.5 cm Petri dishes. On day 68, dishes were photographed and the images transferred to an Apple MacIntosh IIci computer using an Apple scanner and imported into the NIH Image package at a resolution of 150 dpi. Protoplast-derived colonies were identified by thresholding for measurement of total colony area. Mean % area covered and mean colony area (mm^2) were 1.4% and 1.8 mm^2 respectively, for untreated controls, 24.1% and 2.0 mm^2 for Pluronic F-68-supplemented medium, 35.1% and 4.0 mm^2 for perfluorodecalin alone, and 58.5% and 12.5 mm^2 for perfluorodecalin plus Pluronic F-68 supplementation. These results demonstrate a beneficial and synergistic effect of supplementing protoplast cultures with oxygenated perfluorodecalin and Pluronic F-68.

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USE OF DIFFERENT METHODS FOR JUDGING PFC BIOCOMPATIBILITY IN VIVO

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Storage of perfluorochemical compounds (PFC) may lead to functional changes, predominantly in organs with a high part of sessile macrophages. While very acute reactions within the circulation of the lung can be tested by controlling the pulmonary arterial pressure, longer lasting effects must be traced by different methods which test the state of the reticuloendothelial system (RES); the detoxifying function of the liver has to be examined, too.

By means of the clearance of **colloidal carbon particles** from the blood stream, a reduction of the phagocytotic activity is demonstrated by a decrease of the elimination constant k or a prolongation of the half life t/2 of administrated carbon material (which can be detected spectroscopically in hemolized blood).

The vital meaning of a reduced phagocytotic activity is revealed by an **endotoxin test**: compared with the reaction of a control group of animals (mice), the tolerance to a certain endotoxin dose (e.g. escherichia coli lipopolysaccharide) can be distinctly reduced, leading to a significantly increased death rate when no therapeutic agents are given.

Longitudinal studies of the activity of RES cells within the liver can be performed by a **magnetometric method**. In this case, a ferromagnetic γ Fe_2O_3 suspension is given as indicator which is rapidly taken up by cells of the RES. Following magnetization by an external magnetic field, the remnant field above the animal's liver can be measured. The temporal decrease of the field strength above this organ is a measure of the cytoplasmic motility within macrophages, which is largely depressed by incorporated PFC particles. By this method much smaller retardations of the magnetic relaxation after PFCs of the second generation could be shown.

The mean dwell-time of PFCs within tissues with a large content of phagocytotic cells can be determined by gas chromatography in organ samples or noninvasively by ^{19}**F NMR spectroscopy or imaging** in situ. The latter method gives the advantage of longitudinal studies of PFC localisation within the same animal.

Besides functional tests of the state of the liver (activity of certain plasma enzymes and indocyanine green clearance), the **detoxification of pentobarbital** can be repeatedly tested by controlling the sleeping time. Thus the different influence on cytochrome P-450 is revealed.

All these methods distinguish between PFC agents of the first and following generations, thereby indicating any changes in the potential of this treatment.

EFFECTS OF DIFFERENT PERFLUOROCHEMICAL COMPOUNDS ON RETINAL TISSUE

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Three different perfluorochemical (PFC) compounds were tested for their effects on retinal tissue after they had remained within the vitreous cavity of rabbits for two weeks. The PFC agents used were: perfluorooctan, perfluorodecalin and Fluosol-DA 20% emulsion (FDA), a 20% w/v mixture of 0.7 parts of perfluorodecalin and 0.3 parts of perfluorotripropylamine.

The oxidative tissue damage and the inflammatory response of the retina was determined by evaluating the lipid peroxide concentration, expressed by the thiobarbituric acid reaction, and the myeloperoxidase (MPO) activity.

Oxidative tissue damage and inflammatory response of the retinal tissue could be demonstrated. Both parameters showed significant differences in the effects which the various perfluorochemicals had on the tissue as compared to controls.

As has been shown in other cases, the retinal damage is of a mainly mechanical nature, not in itself leading to oxidative alterations. Whereas only FDA showed a significant oxidative damage, the inflammatory activity was significantly increased in all groups. The increased MPO activity and the observed oxidative damage of the retina seem to be the effect of PFC-loaded macrophages and inflammatory induced lipid peroxidation.

INTRAMOLECULAR CROSSLINKING OF HEMOGLOBIN A BY SULFOSUCCINIMIDYL SUBERATE: APPLICATION OF CROSSLINKED PROTEIN AS A BLOOD SUBSTITUTE. B. N. Manjula and A. S. Acharya, Division of Hematology, Albert Einstein College of Medicine, 1300 Morris Park Ave, Bronx, NY, 10461.

The amino group specific reagents with a negatively charged functional group at their distal end exhibit a high degree of selectivity towards the amino groups of the DPG binding pocket, i.e to β-chains of HbA (Acharya et. al. 1994, JBC, **269,** in press). Therefore, sulfosuccinimidyl esters could be targeted to the residues of $\beta\beta$ cleft of HbA and the bis-sulfosuccinimidyl esters of aliphatic dicarboxylic acids could serve as the 'affinity directed' $\beta\beta$ crosslinkers of HbA. The monofunctional reagent, sulfo succinimidyl acetate, as well as the bifunctional sulfosuccinimidyl suberate exhibited a high degree of selectivity to the β-chain of HbA at pH 7.4, in the oxy conformation of the protein. The suberate reacted HbA retained the mass of 64K, just as the acetate reacted material; thereby demonstrating the absence of intermolecular crosslinks in the bifunctional reagent reacted sample. On the other hand, the RPHPLC map of the suberate ester modified HbA is distinct from that of the acetate ester (mono functional reagent) modified product. Besides, the molecular mass of the suberate modified HbA is 64 K even when it is subjected to gel filtration in the presence of 1 M $MgCl_2$ whereas the acetate reacted material dissociated into 32K species just as the unmodified control. The results reflect the introduction of intramolecular crosslinks into HbA by the suberate. The intramolecularly cross-linked Hb has a reduced oxygen affinity (the P_{50} nearly doubled). The decrease in the oxygen affinity of HbA seen on crosslinking with sulfosuccinimidyl suberate is comparable to that seen on formation of HbXL99α, suggesting the potential application of this crosslinked Hb as a blood substitute. The reactivity of the sulfosuccinimidyl esters of tartaric acid and sebacic acid with HbA was also investigated to establish the appropriate length of the alkyl chain optimal for this crosslinking reaction. The optimal length of the 'spacer arm' of the reagent is about 11 A° and is provided by the suberate. The sulfosuccinimidyl ester of suberic acids is a new addition to the class of $\beta\beta$ cross linkers for the preparation of the Hb based blood substitutes.

BLOOD SUBSTITUTES PRODUCED BY CHEMICAL MODIFICATION OF HEMOGLOBIN AND BY MUTAGENESIS

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Requirements for a useful hemoglobin-based blood substitute are that the derivatives have an oxygen affinity that is low enough to permit O_2 to be released readily and that the tetrameric hemoglobin derivative ($\alpha_2\beta_2$) not dissociate into $\alpha\beta$ dimers that would be rapidly cleaved from the circulation. To achieve the first requirement, there are certain regions of the hemoglobin tetramer to which reagents that form covalent bonds can be directed, i.e. the DPG binding cleft between the 2 β-chains. Two examples are acetylation and N-carboxymethylation. Both reactions are mild so that the hemoglobin derivative retains all of its function, i.e. cooperativity. Another approach is to substitute some of the amino acids at certain regions of the tetramer by site-specific mutagenesis to achieve a lowered oxygen affinity. Several mutant hemoglobins have been produced by the yeast recombinant system, including HbA and HbS (1,2,3). The yeast system provides several advantages such as correct N-terminal processing and the utilization of endogenous yeast heme to produce a soluble hemoglobin tetramer that is properly folded. The molecular weight of the purified recombinant sickle hemoglobin is identical to that of natural sickle hemoglobin as determined by mass spectrometry. The data indicate that the primary and secondary structures of the recombinant and natural hemoglobins are the same. Circular dichroism studies show that the tertiary and quarternary structures of the recombinant Hb are virtually identical to those of natural Hb.

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KINETICS OF RECOMBINANT HEMOGLOBIN ASSEMBLY

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The *in vitro* assembly rates of several E. *coli*-derived recombinant hemoglobin molecules were determined under various conditions. Subunits were isolated using methods similar to those described by Bucci and Fronticelli. The recombinant hemoglobins studied included a molecule similar to native hemoglobin and several dialpha-linked proteins having different length glycine linker regions between the C-terminus of α_1 and the N-terminus of α_2. The absorbance change that occurs during these reactions allowed the assembly to be monitored with the use of a spectrophotometer. The rates of assembly were studied under various conditions, including different phosphate concentrations, different subunit concentrations, and non-phosphate buffers. With the proteins that were studied in non-phosphate buffers, the assembly rates were similar to one another. When phosphate was present, the assembly rates decreased with increasing phosphate concentration. Within the range studied, there was no difference in the rates of assembly due to a change in subunit concentrations. These results are similar to control data for assembly of native human hemoglobin.

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DIFFERENTIAL EFFECTS OF PERFLUOROCARBON (PFC) BLOOD SUBSTITUTE EMULSIONS ON LEUKOCYTE ADHESION.

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Due to their enhanced oxygen carrying characteristics, perfluorocarbon emulsions are valuable adjuncts to coronary revascularization procedures. However, the effects of perfluorocarbon emulsions on white blood cell function are unclear. The purpose of this study was to determine the effects of three different perfluorocarbon emulsions on the adhesion characteristics of blood leukocytes. Heparinized blood from donor rats was mixed with either Phosphate Buffered Saline (PBS), PFB-1, PFB-2 (both non-complement activating perflubron emulsions, Alliance Pharmaceutical Corp.) or Fluosol (20% w/v PFC, Alpha Therapeutic) in a ratio of one part emulsion to six parts blood. The blood-emulsion mixtures were incubated for ten minutes at 37°C, then passed down nylon wool leukocyte adhesion columns (1). Blood samples were counted for: Leukocytes, Granulocytes and Lymphocytes. % Cell Adherence was calculated as: {1 - [WBC-out]/[WBC-in]}. We found:

	PBS	Fluosol	PFB-1	PFB-2
Leukocytes	22± 1	23± 1	31± 2*	22± 2
Granulocytes	40± 2	56± 3*	58± 2*	34± 2
Lymphocytes	19± 1	20± 1	26± 2*	18± 2

[Data expressed as % Cell Adherence, Mean ± SEM, *$P<0.05$. ANOVA w/ Scheffe]. For Fluosol and PFB-1, the granulocyte adherence was significantly increased compared to PBS control. In contrast, PFB-2 (designed for reduced cell surface activity) caused no significant change in leukocyte adhesion. In fact, a modest decrease in granulocyte adhesion was observed with PFB-2. These findings suggest a direct effect of some, but not all, PFC emulsions on blood leukocyte function.

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DOES THE PRESENCE OF DEX-BTC-HB INFLUENCE THE IN VITRO RHEOLOGICAL PROPERTIES OF A HEMODILUTED BLOOD ?

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While conducting pharmacology investigation into oxygen carriers, it seemed important to study the *in vitro* and *in vivo* rheological behavior of blood cells in such preparations. However relatively little is known on the topic. With regards to the original nature of human hemoglobin bound to benzene tetracarboxylate substituted dextran (dex-BTC-Hb), it seemed necessary to study its rheological effect in a simulated *in vitro* hemorragic shock compensated for by a blood substitute.

The viscosity of blood substitutes was determined as well as the following rheological parameters after various incubation periods of red blood cells with substitutes: (1) filtration of washed erythrocytes through filters (pore size:3µm) after a contact period of 0, 24, 48 hrs (erythrometer, Sefam, France); (2) viscosity of blood substitute mixtures after a contact period of 0, 3 and 6 hrs at different levels of plasma substitution using a Couette viscometer (Low Shear 30, Contraves, Suisse); (3) erythrocyte aggregation of blood substitute mixtures after a contact period of 0, 3 and 6 hrs by determining the velocity of rouleau formation and the cohesion of rouleau network (erythroagregameter, Affilio, France).

The present work yielded several observations :

-The viscosity of dex-BTC-Hb was slightly higher than those of solutions of native Hb, dex-BTC T10, dextran 40 (Plasmacair ®), modi -fied fluid gelatin (Plasmion ®) and hydroxyethyl starch 200 (Elohes ®).

-Even after an extended contact period of up to 48 hours, the blood cell filtration was not significantly altered.

-The substitution of a larger level of blood substitution with dex-BTC-Hb, corresponding to a compensated 50 % hemorragic shock, slightly increased the viscosity of hemodiluted blood as compared to other substitutes.

- In the presence of dex-BTC-Hb, the aggregation of erythrocytes appears to be increased as compared to standard solutions. Yet the effect was close to that of Plasmion® or Elohes®. In vivo experiments carried out in guinea pigs which had 3/4 of their blood replaced with dex-BTC-Hb revealed that hemodynamic parameters were slightly modified and remained comparable to standard solutions

A dynamic study in adult swine hemodiluted with dex-BTC-Hb is required to study the possible aggregation role of this substitute in vivo and to evaluate its consequences.

CORRELATION BETWEEN PREOPERATIVE HAEMOTRANSFUSIONS AND THE FATE OF RENAL ALLOGRAFTS

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There are contradictory bibliografic data concerning the influence in vivo of blood transfusions (number and time before renal allotransplantation) upon the fate of the kidney allografts after operation. This is the reason the authors of present study to examine possible correlation of preoperative haemotransfusions in several groups of recipients of cadaver renal allografts. The groups are: recipients without haemotransfusions (5 patients), recipients with less than 10 haemotransfusions (32), recipients with 10-25 haemotransfusions (21), recipients with more than 30 haemotransfusions (9). In addition, the titres of anti-T and anti-B-antibodies (warm and cold) are dynamically examined. The rejection crisis and eventual lost of the renal allografts due to severe immune conflict is clinico-laboratorily analysed by a model of immunologic monitoring. As a result of the complex analysis it is concluded that most favourable fate of the renal grafts shows the group of recipients with 10-25 haemotransfusions (last transfusion 9 days prior to operation). Certain positive effect exirt also the anti-B-antibodies (cold) and definite type of immunoreactivity of the patients before renal allotransplantation.

INFLUENCE OF PERFLUOROCHEMICALS ON DETOXICATION ENZYMES OF ANIMALS

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An activation of the liver monooxygenase system [1] as well the phase II detoxication enzymes: UDP-glucuronosyl transferase and glutathione S-transferase [2] is not a single event that takes place in an organism after administration of fluorocarbon emulsion. Other enzymic systems of xenobiotic protection including liver etherases have been also activated. Moreover, administration of the fluorocarbon blood substitute, Perftoran, results in redistribution of organo-phosphorous poisons removing them from sensitive organs to insensitive ones. The liver detoxication enzyme induction by PFCs is followed by an increase of animal resistance to organo-phosphorous pesticides and fluoroacetate and a decrease of that to carbon tetrachloride and triorthocresyl-phosphate. The greatest efficacy of fluorocarbon protective action was demonstrated under a combined use of fluorocarbons and traditional antidotes. An application of PFCs as a new promising drug for prophylaxis and treatment of some liver dysfunctions, poisoning and cancer chemotherapy will be discussed.

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IN VIVO AND *IN VITRO* PERFORMANCES OF CARBOXYMETHYL & CARBOXYMETHYL/GLYCOCHITIN LIPOSOMES ENCAPSULATING HEMOGLOBIN AS POTENTIAL BLOOD SUBSTITUTES

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This study examines the effect of surface charge and hydrophillic surface groups on liposome clearance from blood. Two types of polyanionic chitin derivatives, carboxymethylchitin and another copolymer carboxymethyl/glyco-chitin were used instead of dimyristoylphosphatidylglycerol (DMPG). These derivatives were synthetized at different molecular weights (MW) ranging from 7.0×10^4 to 2.0×10^6. Liposomes with (d_{ave}= 0.15 μm) were prepared by film hydration, followed by high pressure extrusion. The liposome surfaces were then modified by applying the various polymers by physical adsorption. The shear viscosity of the liposome suspensions was compared to that of rat blood at 37 °C. The higher the MW of the polymer used to coat the liposomes the lower was the viscosity of the liposome suspension. The extent of shear-induced leakage was determined at a shear rate of 450 s^{-1}. It was found that liposomes coated with polymers of intermediate molecular weight leaked the least. Half-life measurements of the coated and uncoated (control) liposomes were conducted using normovolemic Sprague Dawley ♂ rats (250-300 g, N=3) . A volume equal to 33% of the blood volume was infused into each rat at 1 $ml.min^{-1}$. When the amount of liposomes was equivalent to a phospholipid dosage of 40 μg/g of body weight there was no difference in half-life between the coated and uncoated liposomes. However, when the amount of the liposomes infused was equivalent to a dosage of 80 μg/g of body weight the coated liposomes remained in circulation for a significantly longer period of time. This suggests that larger doses may be required to detect the effect of the coatings on the circulation persistence of the liposomes.

THE AUTOXIDATION OF ALPHA-ALPHA CROSS-LINKED HEMOGLOBIN: A POSSIBLE ROLE IN THE OXIDATIVE STRESS TO ENDOTHELIUM

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Different types of hemoglobin preparations have been formulated as potential red cell substitutes for a variety of clinical applications. Nevertheless, the apparent toxicity exhibited *in vitro* and *in vivo* by these chemically modified hemoglobins still constitutes the major impediment to progress in the development of a usable blood substitute. The aim of the present study was to investigate the role of hemoglobin autoxidation in the induction of endothelial heme oxygenase (HO), an inducible "stress" protein which is responsible for heme catabolism. We also investigated whether the propensity of different hemoglobins to oxidize correlates to endothelial heme uptake and cell injury. Porcine aortic endothelial cells were incubated for six hours in the presence of 60 μM unmodified hemoglobin (HbA_0), hemoglobin cross-linked between the α chains with bis-(3,5-dibromosalicyl) fumarate (ααHb) or cyanomet-αα-hemoglobin (CNmetααHb). Microsomal HO content increased 3.8-fold in the presence of ααHb, 2.5-fold with HbA_0 and 1.8-fold with CNmetααHb over the control value. The rates of methemoglobin formation exhibited a linear relationship over the time of incubation ($r=0.94$) and the apparent rate constant was 1.8-fold higher for ααHb (0.023 h^{-1}) than HbA_0 (0.013 h^{-1}). In addition, a linear relationship was obtained by plotting the rates of autoxidation of hemoglobins versus the HO activity ($r=0.99$). No further significant increases in HO induction were observed in cells incubated with the oxidized form (100% methemoglobin) of ααHb and HbA_0. Intracellular heme concentration, measured after 24 hours of incubation, was also significantly greater in the presence of ααHb (52.6% over baseline) compared to HbA_0 (10.8%) and CNmetααHb (15.3%) groups ($p<0.05$). However, lactate dehydrogenase (LDH) release, measured as an index of endothelial cell injury, increased in all the hemoglobins examined: ααHb, 33.8±1.1 U/l; HbA_0, 38.5±3.5 U/l; CNmetααHb, 41.9±4.0 U/l; (control group, 19.4±2.8 U/l). We conclude that: 1) the higher rate of oxidation of ααHb renders the molecule more susceptible to induce endothelial oxidative stress (HO induction); 2) the accelerated methemoglobin formation is directly correlated to intracellular HO content and endothelial heme uptake; 3) persistent cell injury suggests that other factors besides heme release may contribute to the hemoglobin-mediated cytotoxicity.

Expression of Recombinant Hemoglobin Rainier

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The naturally occurring Hemoglobin Rainier contains a variant β chain in which the β145 tyrosine is replaced by cysteine. This cysteine is able to form disulfide crosslinks with β93 cysteine giving rise to stable tetramers. Hb Rainier has a high affinity for oxygen, a reduced Hill coefficient, and half of the alkaline Bohr effect, making it a candidate for the treatment of acute hypotension. We report the expression of the individual α and $\beta^{Rainier}$ globins and coexpression of $\alpha/\beta^{Rainier}$ in Saccharomyces cerevisiae using a galactose regulated hybrid promoter. This promoter, containing GAL1-10-UAS and the transcriptional initiation site of the yeast glyceraldehyde 3-phosphate dehydrogenase gene (TDH3) was constructed by PCR. The $\beta^{Rainier}$ mutation was introduced by recombinant PCR. The α and $\beta^{Rainier}$ expression cassettes were cloned separately in a high copy leu2-d yeast vector (pPM40) and introduced into a respiratory proficient yeast strain (JM-43, leu^- ura^-). A 16,000 mol wt band protein (single band) cross-reacting with anti-human Hb antibody was detected on 15% SDS/PAGE gels using the ECL detection system. Densitometric scans of Western blots indicated that each α or $\beta^{Rainier}$ chain represented 3-5% of total protein. Coexpression of α and $\beta^{Rainier}$ was obtained using a single plasmid which contained both α and $\beta^{Rainier}$ expression cassettes cloned at two unique sites. Two bands comigrating with α and β chains of HbA were detected by Western blot six hrs after induction and the proteins accumulated over time. Functional characterization of Hb Rainier was carried out and data will be presented.

IMPROVED RESPONSE TO RADIATION THERAPY IN TUMORS SENSITIZED WITH PEG-HEMOGLOBIN.

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Increasing the oxygenation of tumors has been shown to improve tumor cell kill following radiation treatment. In this study, PEG-Hemoglobin (PEG-Hb) was tested for its ability to deliver oxygen to poorly vascularized tumor tissues. Sprague-Dawley rats were injected subcutaneously with 0.15 ml of cultured tumor cells (osteogenic sarcoma or C6 glioma) at a concentration of 10^8 cells/ml. When tumors had reached 2-3 cm in diameter, the rats were infused with either saline or PEG-Hb (6-25 ml/f, 6%) and tumor tissue oxygen levels were measured. Tissue oxygen tension was measured in the tumor using the phosphorescence decay method (OxyMap™, Medical Systems, NY). Those rats infused with saline showed a tumor oxygenation of 0-5 torr, while rats infused with PEG-Hb showed 2-4 fold increases in oxygenation to levels of 12-16 torr. Maximum tissue oxygenation was reached 2-3 hour post infusion. At two hours post infusion, the rats underwent total body irradiation of 400 Rads Cs at 120 Rads/min for 3.33 minutes. After 2-5 weeks, nine out of ten rats that were infused with saline exhibited continued tumor growth, while greater than 90% of the rats that were infused with PEG-Hb showed significant regression of complete remission of the tumors. This preliminary data indicates that PEG-Hb may be an effective sensitizer for radiation therapy.

RECENT DEVELOPMENTS IN PHARMACOKINETIC MODELING OF PERFLUOROCARBON EMULSIONS

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Perfluorocarbon (PFC) emulsions are potential oxygen carriers. This study is to investigate pharmacokinetic compartment models and physiologically based models, which correlate the distribution of PFC emulsion in the blood, RES organs and non-RES tissues with the excretion data. The models are evaluated by nonlinear regression analysis (using PCNONLIN software) with data of animal with i.v. injection of a concentrated perflubron (perfluorooctyl bromide, PFOB) emulsion. One model with four compartments (representing PFC emulsion in blood, RES organs, non-RES tissues, and PFC per se in blood) and adequate first-order outputs satisfies the mathematical and physical criteria. For this model, the predicted half-lives of perflubron in blood, RES organs and non-RES tissues are 9.4 hours, 5.5 and 14.5 days, respectively, which agree well with the literature half-lives of perflubron in those tissues, which are 3-12 hours (highly dependent on emulsion droplet size), 3-6 days, and 14-20 days, respectively. The physiological modeling provides insight of physiological mechanisms. The relationship between the compartment model parameters (rate constants) and physiological parameters (tissue volumes, flow rates, etc.) is presented. An advantage of physiological model is that prediction may be made in interspecies scaling. The above two kinds of modeling are useful in many applications, e.g. to describe and predict the time course of PFC disposition throughout the body.

LARGE VOLUMES OF LONG-LIVED HIGH-P50 RED BLOOD CELLS LOADED WITH INOSITOL HEXAPHOSPHATE IN A CONTINUOUS FLOW ELECTROPORATION SYSTEM

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Encapsulation of Inositol Hexaphosphate (IHP) in red blood cells (RBC) by a variety of techniques has led to erythrocytes with a low P50 value and to an enhanced capacity for O2 release. Major physiological effects have been observed when transfusions of RBC-IHP were given to piglets The IHP encapsulation methods used were somewhat cumbersome and not well suited for obtaining high volumes of RBC-IHP. The flow electroporation system which we developed includes an exponential pulse generator, a newly designed flow electroporation chamber, a cooling unit and a peristaltic pump. This unit is connected to a plasmaphoresis device which permits the entire procedure to take place in a closed circuit. Typically, 24 hours after electroporation, the P50 values of thr RBC are 50-60 Torr, the lysis is less than 8% and the hematological indices are practically normal. Enhancement of the O2-release capacity of the RBC brings about significant physiological effects in experimental animals (piglets): 1) reduction of the cardiac output, linearly dependent upon the P50 value of the circulating RBC; 2) increase of the arterio venous difference; 3) improved tissue oxygenation. Long term experiments showed that such effects of high-P50 RBC-IHP are maintained over the entire life span of the cells (unchanged as compared to control RBC). Experiments on animals (5-8) and isolated organs (9) show that the O2 release by RBC-IHP is regulated. Toxicology results are presented which indicate an absence of acute or sub-chronic negative effects of RBC-IHP.

MULTI-VARIABLE APPROACH TO THE DESIGN OF CROSSLINKED HEMOGLOBINS

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Computer-aided molecular design is being used to propose new crosslinking reagents for human hemoglobin. In this design process, molecular dynamics calculations have been used to assess the flexibility of both the reagent and the reaction site on the protein. Long, hydrophilic diaspirin reagents have been designed to crosslink different hemoglobin tetramers together or to crosslink hemoglobin to protective enzymes, such as catalase or superoxide dismutase. Multilinkers, which react with the protein at more than two sites, are being made to modify single hemoglobin tetramers and to form hemoglobin octamers. While these modifications depend on the well-characterized aspirin and methyl phosphate alkylation reactions, the sites of reaction can be controlled by the length and flexibility of the reagent. Reagents designed to react between tetramers as they are oriented in the crystalline state are also being used. These reagents offer the possibility of forming complexes that cannot be made in solution. X-ray crystal structures of some of the crosslinked hemoglobins are being determined. Finally, molecular dynamics simulations are being used to predict the effects of crosslinking on protein flexibility. (Supported in part by a grant from the Research Corporation.)

PERFLUOROCARBON EMULSIONS INCREASE TRANSFER OF OXYGEN IN PLASMA FROM ERYTHROCITE TO TISSUE

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The hypothesis about the increasing of O_2 transfer by erythrocytes in the presence of perfluorocarbon emulsions (PFCE) was proposed on the basis of the calculation of O_2 diffusion in the perfluorocarbons and experimental data about the role of PFCE in the O_2 transfer in the blood. The erythrocyte oxy- and deoxygenation kinetics and the influence of the PFCE on the blood were studied using the device modeling blood circulation in the organism. The change of O_2 transport in the erytrocyte-plasma-tissues stage was confirmed. It was shown, that the PFCE increases the oxygenation rate as compared to that of the control blood samples at oxygenation degrees a from 20 to 97%. We have shown ealier that erytrocyte membrane can regulate the O_2 flow from erythrocytes to tissues, changing its nonspecific permeability in thousands times during the cycle of oxygenation/deoxygenation, moreover there are reasons to suppose that cells of other tissues can also regulate their nonspecific membrane permeability depending on their O_2 demand. It has been discovered that the PFCE as well as the emulgators (but in less degree) at the greate a prevent the alteration in the nonspecific permeability. The discovery of PFCE influence on the blood O_2 transport allows to substantiate the possibility of use of PFCE small doses for treatment of some critical (hypaxic) states of the body. However, it is necessary to take into account the PFCE effect on the mechanism of the oxygen membrane permeability change, which can be dangerous for the organs, for which the oxygen abundance is harmful.

INVESTIGATION OF LIPOSOME ENCAPSULATED HEMOGLOBIN /PLATELET INTERACTIONS USING INDIUM-111 LABELED PLATELETS

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A transient thrombocytopenia has been reported in rats after infusion of liposome encapsulated hemoglobin (LEH). Specific quantitation of this thrombocytopenia has been difficult due to interference of LEH with platelet counting by light scattering methods due to an overlap in their size. Specific localization of platelets during this transient LEH induced thrombocytopenia has not been previously described. In order to study the effect of LEH on platelets biodistribution, platelets were labeled with indium-111 and monitered with a gamma scintigraphic camera. Autologous platelets were isolated from 5 mls of venous blood drawn from Sprague-Dawley rats and labeled with 50 microcuries of indium-111-oxine. The platelets were then resuspend in plasma and reinfused into the rats. After a 15 minute equilibration, serial 1 minute gamma camera imaging was started and reinfusion began of either LEH, blank liposomes or free hemoglobin (n=6 per group). Imaging was continued for 2 hours. Serial blood samples were drawn at baseline and at 2, 5, 15, 30, 60, 120 minutes post-infusion. Blood samples were subsequently counted in a well counter to determine platelet levels in the circulation. The blank liposomes and free hemoglobin had no significant changes in the circulation levels of the platelets, while the LEH showed a drop to 50% baseline levels in the indium-111 labeled platelet activity at 2-5 minutes post-infusion with a subsequent gradual return to baseline by 60 minutes. Images were analysed by region of interest placement and curve generation, and data subsequently averaged for each formulation . The images revealed no significant changes in the biodistribution of the platelets over the 2 hour period following infusion of blank liposomes or free hemoglobin. With LEH, the platelet activity rapidly moved out of the circulation and redistributed most prominently to the lungs. An increase in liver activity was also noted. Over a 60 minute period, the activity left the lungs and liver and returned to the circulation. LEH-induced thrombocytopenia is associated with a transient retention of platelets in the lungs and liver. Indium-111 labeled platelets can be used to study mechanisms of LEH-induced thrombocytopenia and aid in the development of LEH formulations not associated with this thrombocytopenia.

USE OF CYCLOTRON PRODUCED OXYGEN-15 TO STUDY OXYGEN CARRYING CAPACITY OF FREE AND LIPOSOME ENCAPSULATED HEMOGLOBIN

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New methods need to be developed to quantitatively assess the oxygen carrying capacity of blood substitutes post-infusion. In this study, we used a new method to study the uptake of oxygen from the lungs. This method utilizes the short lived positron emitting isotope, oxygen-15 (half-life 2.02 min). The oxygen uptake of various hemoglobin based blood substitutes were studied in rats given 40% hypovolemic exchange transfusions relative to the remaining RBC's. Four formulations were studied in 4 rats each: (1) liposome encapsulated bovine hemoglobin (LEBH), (2) liposome encapsulated human hemolysate* (LEHH),(3) free bovine hemoglobin(HEM) and (4) a control saline solution (SAL). Pentobarbital anesthesized rats with previously placed femoral artery and tail vein lines were intubated. 40% hypovolemic exchange transfusions was then performed with each of the formulations listed above. After a 15 minute equilibration time, 3-5 mCi of oxygen-15-O_2 gas was ventilated into the rat and immediate serial arterial sampling in 50 microliter capillary tubes were started and continued for 2 minutes. Samples were then rapidly centrifuged for 4 minutes. RBC, LEH and plasma fractions were separated and counted in a scintillation well counter. The above procedure was repeated for each formulation at 3 hours and 24 hours. The table below shows the oxygen carrying capacity replaced as a percent of the original RBC fraction removed.

	LEBH	LEHH	HEM	SAL
15 min	16%	26%	13%	2%
3 hour	13%	21%	3%	2%
24 hour	3%	15%	1%	2%

LEHH maintained significant oxygen carrying capacity for 24 hours. This prolonged carrying capacity may be due to the met-hemoglobin reductase contained in the LEHH. These studies demonstrate the feasibility of using this method to assess the oxygen carrying capacity of various blood substitutes.

* Human hemolysate provided by the U.S. Army.

Human Hemoglobin-based Blood Substitutes

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The clinical use of human hemoglobin-derived blood substitutes has the potential to be jeopardized by factors other than those inherent to the product. It is essential to show that both the hemoglobin and its modified product are free of contaminants. This is particularly important when attributing pharmacologic effects observed in preclinical and clinical trials to the product or to impurities. Unequivocal demonstration of freedom from infectious agents is mandatory. The product requires detailed characterization such that sites of protein modification are understood and controlled. Proof of safety and freedom from serious side effects, beyond the levels for existing products, are necessary preliminaries to undertake new clinical studies. *In vivo* effects of Hemolink™ on hemodynamic parameters, renal function, immune response and microcirculatory function have been determined. The distribution and rate of product clearance have been characterized. Finally, since red blood cells have established a cost target, blood substitute pricing will have to be competitive. To achieve this, an efficient manufacturing process providing high product yields at relatively low costs is required. Hemosol's product meets all of these criteria.

DEVELOPMENT OF A NEW LIPID-BASED HEMOGLOBIN CARRIER

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The concept of using biodegradable carriers as delivery vehicles for hemoglobin has been pursued by a number of groups. The major driving force for developing hemoglobin carriers is the extension of circulating hemoglobin half-life and reduced toxicities of free hemoglobin. We have developed a novel carrier which is based on the lamellar organization of lipid bilayers into a bicontinuous cubic phase. The lipid employed for these studies is 1-monooleoyl-rac-glycerol. Small angle x-ray scattering studies have shown this phase to have long-range three dimensional periodicity with a cubic symmetry. The cubic phase, which appears macroscopically as a viscous gel, contains nanosize pores whose size can be precisely controlled. It has been shown that the cubic phase can entrap high percentage of proteins without loss of the cubic structure. The cubic phase can be processed into sub-micron sized particles referred to as cubisomes. The high encapsulation efficiency and the possibility of processing cubisomes so that they can easily be sterile filtered, makes them attractive candidates for hemoglobin encapsulation. We present different methods employed to make the cubisomes with encapsulated hemoglobin. X-ray diffraction results indicate that high concentrations of hemoglobin can be entrapped without the loss of the cubic structure. The stability of the cubic phase with entrapped hemoglobin has been examined with regard to hemoglobin retention, methemoglobin formation, and P_{50}. Preliminary toxicity studies of the lipid in mice have been accomplished.

SYSTEMIC RESPONSES TO SFHS-INFUSION IN HEMORRHAGED DOGS

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Anesthetized dogs were prepared for monitoring various organ functional parameters, as described in the accompanying abstract. Removal of 35 ml/Kg of blood resulted in very marked drop in arterial blood pressure (diastolic pressure falling to an average of 29±3 mmHg immediately after bleeding) with subsequent progressive but incomplete recovery. A similar pattern was seen in cardiac output (maximal fall to 37% of control value). Very significant decrements (to ‹50% of control value) in organ blood flow were seen in spleen, kidney, pancreas and gut and lesser falls were seen in the heart, whereas brain and liver (hepatic arterial) flows did not decline. One group of dogs (n=6) was transfused with its own blood (Gr. I) while a second group received SFHS (Gr. II). When compared to reinfusion of the shed blood, the response to the infusion of SFHS was significantly different in some respects. Bradycardia was only seen in dogs of Gr. II. Whereas the recovery of systolic blood pressure was comparable after the reinfusion of blood or SFHS, there was an overshoot in the diastolic blood pressure ($p<.05$) in Gr. II only. Overshoots of comparable magnitude occurred in both groups in left and right atrial, as well as in pulmonary arterial pressures. The recovery of the cardiac output was comparable although it was less well sustained after SFHS. Myocardial blood flow rose above baseline levels in both groups, with no significant difference between the responses. Cerebral blood flow rose higher and the response in hepatic arterial flow was less after SFHS administration. The flow-responses were comparable in the kidney, spleen, pancreas and gut in the two groups. SFHS was rapidly excreted in the urine in which large increases in N-acetyl-glucoseaminidase activity were observed. These experiments represent the appropriate baseline for comparison of responses to modified hemoglobin preparations. [Supported by the Defence and Civil Institute of Environmental Medicine of Canada.]

UPDATE ON PERFLUOROCHEMICAL DEVELOPMENT

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The perfluorochemical emulsion Oxygent™ (Alliance Pharmaceutical Corp.) consists of perflubron (perfluorooctyl bromide [PFOB]) emulsified in egg yolk lecithins, which has been under development for some time as a temporary oxygen carrier for use during high blood loss surgery. The use of this agent will improve tissue oxygenation and hence it may be regarded as an "anti-hypoxic" agent. It will thus allow for more aggressive intraoperative hemodilution without compromising the margin of safety and, hence, reduce allogeneic blood transfusion requirements. Following satisfactory preclinical safety and efficacy studies, clinical trials were commenced in 1992. Phase I safety trials have been completed in healthy volunteers. Phase I/II safety trials have also been completed in anesthetized subjects undergoing surgery, the intended patient population. No major safety issues were observed in these trials and this has allowed commencement of Phase II clinical trials in patients undergoing high blood loss surgery. Computer simulation has predicted that a small dose of Oxygent may be able to allow substantial blood loss to occur while maintaining tissue oxygenation at or above normal levels. These predictions have been validated in a canine model of continuing hemodilution. This data was used to support design of the Phase II efficacy trials, results of which confirm both the computer predictions and correlate with the preclinical data.

INCREASED TUMOR OXYGENATION AND RADIATION SENSITIVITY IN TWO RAT TUMORS BY A HEMOGLOBIN-BASED-OXYGEN CARRYING PREPARATION

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The rat 13762 mammary carcinoma and the rat 9L gliosarcoma were grown subcutaneously in a hind limb of female, Fisher 344 rats. The oxygen content of the tumors was determined using an Eppendorf pO_2 histograph. Fifty-to-sixty oxygen measurements were made per tumor and there were 8-to-10 animals per group. The percent of pO_2 readings $\leq$ 5 mmHg in the mammary carcinoma was 49%, this was decreased to 27% by administration of the hemoglobin preparation (8 ml/kg) and further decreased to 14% when carbogen (95% O_2/5% CO_2) breathing was added to administration of the hemoglobin preparation. The percent of pO_2 readings $\leq$ 5 mmHg in the gliosarcoma was 71%, this was decreased to 62% by administration of the hemoglobin preparation and further decreased to 58% when carbogen breathing was added to administration of the hemoglobin preparation. Therapeutic response was assessed over a single-dose range of radiation therapy (10, 20 and 30 Gray). The dose modifying factor produced by the hemoglobin preparation/air was 1.5 and by the hemoglobin preparation/carbogen was 2.7 in the rat 13762 mammary carcinoma. The dose modifying factor produced by the hemoglobin preparation/air was 1.6 and by 2.9 in the rat 9L gliosarcoma. Hypoxia protects malignant cells from the cytotoxic actions of radiation therapy. These results indicate that administration of a hemoglobin-based oxygen carrier can reduce tumor hypoxia and increase tumor response to radiation therapy. [Supported by NIH grant P01-19589 and a gift from Biopure Corp., Boston, MA.]

CYTOKINE RESPONSE AND VASOACTIVITY FOLLOWING ADMINISTRATION OF LIPOSOME ENCAPSULATED HEMOGLOBIN

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Liposome encapsulated hemoglobin (LEH) has been shown to accumulate in the monocytic phagocyte system (MPS). Initial studies have focused on the production of cytokines following LEH administration in vivo and the effect of LEH on macrophage function in vitro. Injection of 10-25% LEH in mice generates a significant increase of IL-6 at 6-8 hours post-injection, with no increase in TNF. *Ex vivo* phagocytes from animals injected with LEH show reduced levels of TNF following challenge with 1 ug LPS at 1, 2, and 4 hours. Studies with murine macrophage cell line have been used to examine the dose and time response of TNF production following exposure to LEH and LPS simultaneously and sequentially. These results show that the early expression of TNF (1-6 hours) is reduced in samples containing both LEH and LPS. LEH uptake is maximal over the same time period in which TNF expression is significantly depressed (1-6 hours). We are currently examining the mechanism of this response.

We have also examined the interaction of LEH with endothelial cells and an isolated aortic ring model. Vasoactivity of LEH has been evaluated in an isolated rabbit thoracic artery. Carbachol induced relaxation of vessels pre-contracted with norepinephrine was measured in the presence of free tetrameric hemoglobin and in LEH. LEH showed reduced inhibition of relaxation compared to an equivalent concentration of unencapsulated free hemoglobin. This finding could suggest that encapsulation may alleviate some of the observed vasoactive responses of hemoglobin.

LIPOSOME ENCAPSULATED HEMOGLOBIN: MACROPHAGE PHAGOCYTOSIS AND PRODUCTION OF TUMOR NECROSIS FACTOR.

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Tissue resident macrophages are largely responsible for the removal of liposome encapsulated hemoglobin (LEH) from the vascular compartment. One important issue facing the utilization of LEH as a blood substitute is the effect of large dose application on the reticuloendothelial system and secondary septic challenge, a likely military scenario. In our present studies we have focused on the macrophage line RAW 264.7, to further define the interaction of LEH with macrophages and the effects of LEH exposure on cytokine response following secondary challenge. In addition, we have examined the kinetics of LEH phagocytosis. RAWs were cultured with LEH at 3 concentrations (10mg lipid:5mg Hb/ml, 1.0mg lipid:0.5mg Hb/ml, and 0.1mg lipid:0.05mg Hb/ml) for 24 hours. At 24 hours the cells had taken up .15,.06, and .02 percent LEH respectively. We have also measured TNF production by RAWs exposed to varying concentrations of LEH and then challenged with LPS. In agreement with previous findings with other macrophages (1-3), RAWs do not produce TNF in response to incubation with LEH. Preliminary results with co-incubation of LEH and LPS, and measurement of TNF at 1,2,4,6, and 24 hours indicates that LEH may retard the kinetics of TNF production and this may correlate with the rate of uptake of LEH by phagocytosis.

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A COMPARATIVE STUDY OF THE ACCURATE MEASUREMENT OF ENDOTOXIN IN LIPOSOME ENCAPSULATED HEMOGLOBIN.

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An important parameter for the successful large scale production of liposome encapsulated hemoglobin (LEH) is the accurate measurement of endotoxin. We have examined three different methods of endotoxin determination utilizing the Limulus Amebocyte Lysate (LAL) assay to accurately determine endotoxin levels in LEH, 1) gel-clot method, 2) chromogent, a spectroscopic based LAL test, and 3) turbidimetric method which determines endotoxin levels in solutions based on the time needed to reach a specific degree of turbidity. Both the chromogent and turbidimetric methods require significant dilution of the LEH preparation before accurate measurement can be made. We have tested these methods by the addition of known amounts of endotoxin to LEH samples. A comparison of the accuracy of the three methods will be presented. The bioactivity of the detected endotoxin as measured by the production of tumor necrosis factor (TNF) from a murine macrophage (RAW 264.7) has also been measured.

A HYPEROSMOLAR OXYGEN YIELDING BLOOD SUBSTITUTE FOR CARDIOPULMONARY BYPASS

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Conventional cardiopulmonary bypass (CPB) is associated with progressive anasarca and hypoxic acidosis. The former is reduced by hetastarch, mannitol, furosemide, albumin and blood. The latter can be masked by sodium bicarbonate and by induction of ventilatory alkalosis. An oxygen yielding blood substitute capable of reducing anasarca would be better. We subjected 36 large (68 ± 10 kg) nubian cross ungulates to 5.2 ± 0.9 h of CPB. Fluid retention was 25 ± 3 ml/kg/h using normo-osmolar crystalloid prime (303 ± 10 mOsm/l). With the same solution plus mannitol, hetastarch, and furosemide fluid retention was 16 ± 4 ml/kg/h. With hyperosmolar prime alone (828 ± 38 mOsm/l), it was 11 ± 2 ml/kg/h. In a large animal using hyperosmolar prime with 20% to 80 % weight to volume perfluorocarbon emulsion (3.0 l), we found significant reduction in fluid retention and significant elevation of brain tissue oxygenation as assayed by ion selective electrodes. Brain tissue PO_2 was 37 mm Hg in the control animal (normo-osmolar crystalloid prime) vs 169 mm Hg in the hyperosmolar oxygen yielding blood substitute animal. Fluid retention was 27 ml/kg/h in the control animal vs 11 ml/kg/h in the blood substitute animal. We also found that a large portion of the emulsion can be removed from the animal at the end of bypass, making CPB a particularly attractive mode for a perfluorocarbon blood substitute. Conclusions: A hyperosmolar oxygen-yielding blood substitute is feasible for cardiopulmonary bypass to enhance brain oxygenation and diminish anasarca. It decreases anasarca more than normo-osmolar crystalloid prime combined with hetastarch, mannitol, and furosemide. A large portion of the perfluorocarbon can be retrieved post-operatively.
Reference: U.S. Patent .5,114,932 Hyperosmolar Oxyreplete Hemosubstitute.

THE SIDE REACTION CAUSED BY THE PERFLUOROCARBON EMULSIONS IN INTRAVENOUS INFUSION TO EXPERIMENTAL ANIMALS

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The perfluorocarbon (PFC) emulsions are infusion gas-transporting media and are usually called the artificial blood. However anaphylactoid reactions were obserwed during intravenous infusion of PFC emulsions. The cause of side reactions is the activation of the complement system. An indirect sign of the intravascular complement activation is the neutropenic effect in the peripheric blood. Taking into consideration the phenomen,the method of evaluation of anaphylactoid reaction caused by PFC emulsions has been elaborated using the neutropenic index. We evaluated the biocompatibility of 25 PFC emulsions of different composition in intravenous infusion to rabbits using this index. Emulsions evoked reactions of different extent. Some emulsions did not cause the neutropenic effect at all. Results of this work allow to reach conclusion, that influence of PFC emulsions on the complement system is explained not only by emulsifying agent procsanol:the stability of emulsions in the vascular bed and in the period of storage in frozen state is of great importance as well.

STUDY OF POLYMERIZED HEMOGLOBIN IN EXPERIMENT

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Lyophilized polymerized hemoglobin has no group specificity and does not damage the kidney after 2 years of storage; its T/2 is 14-16 hours. P_{50} of solution prepared on the hemoglobin basis is 24-28 torr at 37°C and CO_2, 40 torr in 0.05 M Tris-buffer, pH 7.4. As was shown in models of hemorrhagic shock in dogs, the solution has hemodynamic properties, is capable of increasing the cardiac output and maintains the arterial pressure at the level close to the initial one. Analysis of the oxygen expenditure suggests the expediency of using the solution in doses of 1 gr/kg of body weight. The increase in oxygen capacity of the solution administered in the said dose is the result of increase in both the hemoglobin concentration in blood plasma and amount of circulating red blood cells in the vascular bed at the expense of their release into circulation from the depôt. Contribution of the solution to oxygen uptake by tissues is 18-15%. The solution in the said dose stimulates erythropoiesis. The solution of lyóphilized modified hemoglobin can be used for correction of both hemodynamic disorders and decreased oxygen delivery in hemorrhagic shock.

UNCOUPLING LIVER MONOOXYGENASE SYSTEM BY PERFLUOROCHEMICALS *IN VIVO*

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Perfluorochemicals (PFCs) including those used as a base of blood substitute emulsions, form an enzyme-substrate complex with liver microsomal cytochrome P-450 that is followed by various alterations of monooxygenase system functions. First of all, due to chemical inertness, PFCs appear to uncouple the microsomal hydroxylation that results in the enhancement of NADPH oxidation rate. To evaluate the physiological significance of the phenomenon *in vivo,* 10% vol/vol emulsion of perfluorodecalin (PFD) was administrated to rats and rabbits at a dose of 5 ml/kg. The microsomes isolated from PFD-treated animals demonstrated 2-fold increase of the NADPH oxidation rate that was caused by the PFD location in the active center of cytochrome P-450. Nevertheless, the levels of reduced pyridine nucleotides in liver as well as the glucose concentration in blood were unchanged in a course of experiments. Evidently, the rate of NADPH synthesis by means of malic-enzyme and corresponding dehydrogenases of pentoso-phosphate shunt is much greater than that of NADPH oxidation in the liver monooxygenase system uncoupled by PFC. To demonstrate the PFC uncoupling effect *in vivo* we measured the body weight loss of starved animals. The weight loss in PFD-treated animals was significantly higher (17%; $P < 0.05$) than in control groups. Thus, the uncoupling of liver monooxygenase system by PFCs does not result in considerable alterations in normal healthy animals. Another situation can apparently occurr after PFC blood substitute administration under poisoning or liver pathology/conditions. Application of PFC uncouplers of the liver monooxygenase system as an anti obesity treatment has been discussed.

ENZYME ACTIVITY OF THE CRAB HEPATOPANCREASE COLLAGENASE IN CONCENTRATED FLUOROCARBON EMULSION (FLUOROGEL)

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Fluorochemical emulsion (FCE) well-known as components of blood substituting solutions today are also considered as a promise base for cosmetics and medical ointments. It was suggested that a combination of FCE and some natural biologically active compounds could change the properties of both parts of the mixture. An enzymatic activity of the crab hepatopancrease collagenase in FCE has been investigated. Composition of the preparation studied was as follow:

Perfluorochemicals	55 ml
Proxanol 268 (analog of Pluronic F68)	5 g
Glycerol	0.8 g
D-Sorbitol	1.6 g
1,2 -Polypropylene glycol	0.5 g
Vitamin E	0.5 g
Vantol	0.05 g
Collagenase	0.05 g
Water	to 100 ml

Emulsion particle diameter 150 nm

Two-fold activation of the enzyme in fluorogel has been found. Moreover, in fluorogel the enzyme retains activity in contrast to that in solution. The control experiments have shown that the proxanol activates the enzyme whereas a fluorocarbon phase of the emulsion provides the conservation of the enzyme native form.

OXYGEN TRANSPORTING ABILITY AND BIOCOMPATIBILITY OF ARTIFICIAL RED CELL (ARC)

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The ARC has been prepared by encapsulating purified human Hb in liposome, the membrane of which was stabilized by polymerization of membrane itself. Liposomal encapsulated Hbs have many advantages, such as lower viscosity, lower oncotic pressure, possibility of higher Hb concentration, and also possibility to co-encapsulate co-effecters (such as arosteric factor,enzymes, etc.), comparing with non-encapsulated Hbs, such as cross-linked, polymerized or PEG conjugated Hb etc., because of their larger particle sizes. Furthermore, polymerization of membrane brought us great stability of liposome, during its preservation period and also in blood stream (half life = 21hrs, at 40% exchange rate in transfusion test by using dogs).

Its efficacy and biocompatibility were evaluated under pre-clinical test.

The results of evaluation showed that ARC has sufficient oxygen transporting ability, comparable with native red blood cell, and that ARC was basically biocompatible product which could be used as one of red blood cell substitutes.

PROTECTIVE EFFECT OF SELENIUM ON HEMOGLOBIN MEDIATED LIPID PEROXIDATION *IN VIVO*

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The toxicity of hemoglobin (Hb) solutions is related, at least in part, to the generation of oxygen free radicals. In addition, reaction of the Hb with H_2O_2 forms ferryl-Hb species that initate lipid peroxidation. Previous work from our laboratory has shown that oxidative stress associated with the injection of Hb needed to be controlled; this could be partially accomplished by pretreatment with antioxidants such as vit.E or concomitant injection of oxygen radical scavengers such as mannitol. The present study was designed to examine whether selenium (Se) may prevent oxidative damage observed after Hb administration. Three groups of Sprague-Dawley rats (300-350 g) were compared; (I) the negative control group without treatment; (II) the positive control group with replacement of 40% total blood volume (TBV) with purified modified bovine Hb solution (THb 7 g/dL, M.W. <400kDa); and (III) the experimental group which received dietary supplemented selenium (Na_2SeO_3) in daily doses of 5 $\mu g\ kg^{-1}$ body weight in drinking water, 4 days before and 3 days after administration of Hb solution in the same volume as in group II. In addition, 24 hrs prior to infusion of Hb, this group was injected with single I.M. dose (5 $\mu g\ kg^{-1}$) of Na_2SeO_3. Three days after Hb injection, all animals were sacrificed. Oxidative stress was determinated by measuring MDA, an end product of lipid peroxidation. MDA was measured by the thiobarbituric acid reaction in plasma and homogenates of the perfused liver, kidneys, heart, lungs and brain and expressed as nM of MDA in mL of plasma or gram of wet tissue. The activity of SeGSH-Px in the plasma was used as an indication of selenium status and was determinated spectrophotometrically on the basis of monitoring of the oxidation of NADPH at 340 nm, using H_2O_2 as a substrate. Activity was expressed in units per mL of plasma. Simultaneously, injury-dysfunction of vital organs was assessed by the measurement of plasma LDH (U/L), SGPT (I.U./L), creatinine (mg/dL) and blood P_aO_2 (mm Hg). The results have shown that exchange transfusion of 40% TBV with Hb solution introduced a significant increase of MDA level in the liver (43.5%, $p<0.001$), heart (40.5%, $p<0.05$) and plasma (126.5%, $p<0.001$) as compared to the control animals. No significant changes in the MDA level were observed in the lung, kidney or brain tissue. The levels of SGPT and LDH were elevated, while creatinine and SeGSH-Px remained normal. Arterial blood PO_2 was similar to that of the control. Selenium treatment was very effective in prevention of oxidative damage introduced by Hb. Full protection from peroxide formation was noted in liver tissue. In fact, the liver level of MDA in the Se-treated group was lower (47%, $p<0.001$) than that of control Hb group (II). Also, plasma levels of MDA, SGPT and LDH were significantly decreased and appeared similar to that of the control group (I). Treatment with Se increased the SeGSH-Px level (18.5%, $p<0.1$). The antioxidant activity of selenium can be essential in defence against oxidative stress associated with transfusion of Hb-based blood substitutes.

Polyethylene Glycol Conjugated Bovine Hemoglobin (PEG-Hb) As a Blood Substitute. Robert G.L. Shorr, Ph.D., D.I.C. Enzon Inc., Piscataway, NJ.

Desirable features of a cost effective blood substitute are availability, ease of manufacture, and acceptable shelf life, as well as documented safety and efficacy in human clinical trials. We have chosen to develop polyethylene glycol conjugated bovine hemoglobin (PEG-Hb) as an oxygen carrier due to the ready availability of bovine hemoglobin, its superior oxygen carrying capability and the well documented use of PEG to prolong vascular retention time as well as blunt immune or allergic reactions.

The preclinical efficacy of PEG-Hb has been established in a number of animal models in which the oxygen-carrying ability was variably compromised. In extreme exchange transfusion studies in pigs, where hematocrits were taken to less than 8%, survival was 100% with PEG-Hb and 25% with dextran controls. PEG-Hb was able to oxygenate tumor tissue (C6 glioma and osteogenic sarcoma) to near normal tissue levels with a complete regression response to radiation of >90%. Controls which received lactated Ringers or carbon monoxide saturated PEG-Hb followed by radiation showed a typical partial/complete response rate to radiation of <20%. PEG-Hb has been examined for toxicity in exchange transfusion and volume overload models in mice, rats, rabbits, dogs and pigs. Hemoglobinuria at less than 1% of the total administered dose was consistently observed across species and models in which native hemoglobin caused significant hemoglobinuria. Histological studies showed minor vacuole formation in the kidney suggesting some activation of the RES for PEG-Hb clearance.

FUMARATE-CONTAINING BLOOD SUBSTITUTES OF ANTI-HYPOXIC ACTION

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Measures of battle against oxygen deficiency include blood and blood substitute transfusions. However, improved O_2 transport is effective till the mechanisms of cellular O_2 utilization remain undamaged. Under these conditions, administration of antihypoxant-containing blood substitutes becomes pathogenetically substantiated. Antihypoxic properties of intermediates of Crebs' cycle (succinate, malate, fumarate, oxaloacetate) were studied in models severe blood loss in rats and rabbits. Fumarate appeared to be most effective. Inclusion of fumarate into composition of colloid and saline blood substitutes considerably increased the therapeutic efficacy of these solutions. The latter became capable of restorating the cellular processes of oxidative metabolism under conditions of insufficient oxygen supply to tissues,eg, in the systemic O_2 transport which does not exceed 50% of the initial level the processes of generation of energy in the hepatic mitochondria in fumarate infusion returs to the normal. There was also a correction of the acid-base state (blood pH increased from 7.19 to 7.34; BE decreased from -16.0 to -9.7). On the basis of experimental data, colloid and saline blood substitutes containing fumarate have been developed. A saline blood substitute Mafusol is successfully used in clinics for treatment of shock, hemorrhage, traumas, burns, intoxications as well as for priming the apparatus of artificial blood circulation. Mafusol has been patented in Russia.

INTERACTION OF ULTRAPURE HEMOGLOBIN (Hb) WITH RENAL EPITHELIAL CELLS

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Using an ultrapure, endotoxin-free, dimeric Hb solution, we investigated the cellular processing and mechanisms of Hb toxicity in both cultured opossum kidney (OK) cells and in kidneys from rats infused with Hb. To determine in vivo nephrotoxicity, we infused rats with 1.0-7.5 g/kg of Hb. Acute renal failure (ARF) followed Hb infusions of 7.5 g/kg with mean serum creatinine values at 48 hrs post infusion of 3.4 ± 1.02 mg/dl returning to baseline values of 0.50 ± 0.03 mg/dl by 10 days ($p<0.02$). No ARF was found in control saline infusions of identical volume or in Hb infusions < 5.0 g/kg. Having established parameters of Hb mediated ARF, we studied the effects of Hb on renal epithelial cell endocytosis and membrane integrity. Using spectrofluorometry, we demonstrate that uptake of fluorescein derivitized Hb into renal proximal tubule epithelial cells is by fluid-phase endocytosis and that Hb does not disrupt the membrane integrity of the Hb-laden endosomes as measured by their membrane proton permeability, a sensitive assay of membrane disruption. Since several models of ARF including obstruction and ischemia demonstrate change in both Na/K ATPase activity as well as mRNA expression of its constituent alpha and beta subunits, we quantified changes in subunit expression after Hb-ARF using northern analysis in renal cortex (C) and medulla (M) after normalizing using beta actin. Expression of both subunits in C and M decreased significantly from baseline 24 hrs post Hb infusion. This pattern of change in Hb-ARF was distinct from that manifest by renal injury produced either by ureteral obstruction or renal ischemia. To quantify alterations in renal cortical ATP content produced by Hb infusions, we infused rats with 7.5 g/kg of Hb and performed in vivo ^{31}P nuclear magnetic resonance (NMR) spectroscopy. Whereas ischemic rats display a prompt and sustained fall in ATP levels, Hb infused rats showed no significant changes from baseline. Our data demonstrate that: 1) Large quantities of ultrapure Hb are required to induce Hb-ARF in rats; 2) Hb-ARF does not arise from perturbations in endosomal membrane integrity; 3) Hb-ARF alters Na/K ATPase subunits in a unique fashion; 4) Hb infusion does not alter acutely renal cortical ATP content in the setting of Hb-ARF.

Perfluorocarbons in the Twenty-first Century: Clinical Applications As Transfusion Alternatives

The risks of allogeneic transfusion are well known to physicians and have prompted a search for alternatives. Perfluorocarbons were introduced into clinical trials in the early 1980's with the hope that these products would develop into acceptable blood substitutes. Unfortunately, the limited potency, short half-life, and potential toxicity of these early formulations coupled with unrealistic expectations for efficacy prevented the perfluorocarbons from playing a significant role in transfusion medicine. Recent changes in formulation to improve efficacy and eliminate toxicity have stimulate renewed interest in perfluorocarbons as alternatives to allogeneic transfusion. Our recent work has focused on the role of perfluoroctylbromide (PFOB), a second generation perfluorocarbon, as an adjunct to autologous transfusion and acute normovolemic hemodilution (ANH), rather than as a total blood substitute. Initial animal experiments have shown the ability of small doses of PFOB to maintain oxygen delivery without the need for blood transfusion in the setting of hemodilution. Our presentation will focus on subsequent clinical work using PFOB as an alternative to both allogeneic and autologous blood transfusion during ANH. We believe that perfluorocarbons such as PFOB will have a significant role in the future as one of several, additive alternatives to blood transfusion.

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THE DEVELOPMENT OF RECOMBINANT SYSTEMS FOR STUDIES OF HEMOGLOBIN STRUCTURE AND FUNCTION.

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The protein engineering group at Somatogen has produced a recombinant human hemoglobin that is in clinical testing for use as a cell-free blood substitute. This protein (rHb1.1) has been produced in *Escherichia coli* and in the yeast *Saccharomyces cerevisiae*. Both microbial expression systems yield soluble, correctly folded hemoglobin . Expression in yeast yields an authentic human hemoglobin whereas the protein produced by*E. coli* retains amino-terminal methionine. We have modified human hemoglobin by the introduction of a mutation in the beta-globin gene that results in a substantial increase in the oxygen dissociation rate. The resulting oxygen equilibrium curve is similar to that of human hemoglobin contained in an erythrocyte. A second alteration in the structure of the hemoglobin tetramer results from the fusion of two alpha-globin genes to produce a single gene that expresses a polypeptide containing two alpha-globin domains separated by a short "spacer" sequence. The alpha-globin fusion results in the formation of a pseudo-tetrameric form of hemoglobin that cannot dissociate into alpha/beta dimers, the consequence of which is an increased intravascular half-life of the cell-free protein as measured in rats and in humans. We have also introduced additional mutations that allow docking of two psuedo-tetramers to form a 128 Kd protein and also serve as sites for the reversible coupling of small molecular weight bio-organic compounds.

COMPLEMENT CONSUMPTION, HEMOLYSIS AND THROMBOXANE SECRETION FOLLOWING INJECTION OF HEMOGLOBIN-CONTAINING LIPOSOMES IN RATS

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Liposome-encapsulated hemoglobin (LEH) was administered intravenously to rats (10% top-load) and plasma samples were taken at intervals from 10 up to 180 min after injection. These samples displayed increased free Hb and thromboxane B_2 levels, decreased complement hemolytic titers and increased lytic activity on glucose-containing liposomes in an *in vitro* assay. These changes were maximal at 10 min and decayed thereafter. *In vitro* studies showed that traces of phospholipase A_2 activity, present in the hemoglobin (Hb), were amplified 20 to 300-fold when co-encapsulated with Hb in liposomes. Thin-layer chromatography of phospholipids extracted from empty liposomes and LEH revealed traces of lysolecithin in both preparations, which, along with other products of phospholipid degradation, increased upon incubation at 37°C. This increase was more expressed in LEH than in empty liposomes. Lysolecithin perturbs biological membranes, it induces complement activation (through binding of C-reactive protein) and has vasoregulatory activities *in vivo*. Thus, our *in vitro* findings raise the theoretical possibility of a causal relationship between lysolecithin production and the above described and/or other previously reported transient hemodynamic and hematological effects of LEH in rats. Experiments are currently underway to determine the role of lysolecithin in these *in vivo* effects.

CHARACTERIZATION OF *NEO RED CELLS(NRC),* THEIR FUNCTION AND SAFETY IN VIVO TEST.

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We have developed and evaluated a new generation of red cells, the Neo Red Cells(NRC) as oxygen carriers with metHb to Hb conversion enzyme system being preserved. Stroma free hemoglobin(SFH) prepared without damage to the enzymes from outdated human red blood cells, together with inositol hexaphosphate as an allosteric effector, NAD as a coenzyme and glucose-6-phosphate as a substrate were encapsulated in liposomes composed of hydrogenated soy phosphatidylcholine, cholesterol, myristic acid and α-tocopherol in the ratio of 7:7:2:0.28 respectively. NRCs thus prepared had a mean diameter of 220nm, encapsulation efficiency of 1.3g-Hb:1g-lipid and $P_{50}O_2$ of 45mmHg were then coated with polyethylene glycol bound to hydrogenated soy phosphatidyl-ethanolamine as a surface modifier to prevent aggregation of NRCs in plasma. The oxygen transporting capacity of NRCs was investigated in rabbits which were made anemic by drawing 85% of their blood and replaced it with an equal volume of NRC solution. Their conditions and recovery from anemia was compared with rabbits infused with normal rabbit blood. The rabbits received NRC recovered from anemic condition within 2hr after exchange transfusion and recovered to pre-exchange conditions within 10hr. In this model, whereas oxyHb to metHb conversion was over 35% in the absence of metHb to Hb conversion enzyme system 24hr after transfusion, it was reduced to below 15% when the enzyme system was carefully preserved. The recovery of own red cells began within 24hr following exchange transfusion and full recovery was observed within 2weeks and the animals continued living normally for over 6months before being sacrificed. Further the hemorrhagic shock model rabbits infused with NRCs recovered from acidosis and renal dysfunction faster than rabbits infused with normal rabbit blood. The present investigations suggest that the NRCs with enzymatic reduction system restrained the formation of metHb and they are efficient oxygen carriers without causing serious adverse reactions.

AN OVERVIEW ON OXYGEN-CARRIERS IN CANCER THERAPY

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Solid tumors are aberrant tissues composed of stroma and malignant cells. Amongst the properties which distinguish normal tissues from solid tumors are physiological characteristics related to the disregulated proliferation of the neoplastic and normal cells that comprise the tumor mass. Although signals for vascular growth are present in solid tumors, the growth of blood vessels in tumors is irregular, and the vasculature is often poorly formed and inadequate lacking vasoresponsive elements. Thus, solid tumors often exhibit highly heterogeneous oxygen tension distributions, pH, glucose delivery and utilization, etc. The importance of oxygen to the cytotoxicity of radiation therapy has been recognized since the 1950's and the importance of oxygen to the cytotoxicity of many anticancer drugs has been recognized since the 1970's. Laboratory studies established that the hypoxia in solid tumors provides a protection to viable malignant cells from these therapies. Numerous strategies to oxygenate tumors have been tested in preclinical solid tumor models and those oxygenating methods which succeeded in decreasing tumor hypoxia also succeeded in increasing the response of the tumor to therapy. pO_2 Measurements made in patients have established that therapeutically significant levels of hypoxia exist in human tumors. The intravenous administration of an oxygen delivery agent such as a perfluorochemical emulsion/oxygen or a hemoglobin preparation is the most generally clinically applicable method for increasing tumor oxygen content developed thus far. Increased tumor cell killing and increased tumor growth delay can be achieved with both radiation therapy and chemotherapy without increased toxicity when oxygen carrying agents are used as therapeutic adjuvants. The clinical trial of these materials in cancer is warranted.

INCREASED TUMOR OXYGENATION AND RADIATION SENSITIVITY IN THE LEWIS LUNG CARCINOMA AFTER A PERFLUBRON EMULSION/CARBOGEN AND ANTIANGIOGENIC AGENTS

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The Lewis lung carcinoma growing subcutaneously in the hind-leg of male C57BL mice is very hypoxic having 92% of the pO_2 measurements ≤ 5 mmHg as determined with a polarographic oxygen electrode. Administration of a perflubron emulsion (8 ml/kg) along with carbogen breathing increased the tumor oxygen level so that 82% of the pO_2 readings were ≤ 5 mmHg. Treating tumor-bearing animals with TNP-470 (30 mg/kg, s.c.) on alternate days and minocycline (10 mg/kg, ip) daily beginning on day 4 resulted in decreased hypoxia in the tumors on day 9 when pO_2 measurements were made. The percent of pO_2 readings ≤ 5 mmHg in the TNP-470/minocycline treated was 75% which upon administration of the perflubron emulsion along with carbogen breathing was reduced to 45%. Therapeutically daily fractionated radiation (2, 3 or 4 Gray x 5) was used as an oxygen-dependent cytotoxic modality. The radiation response of the tumors in TNP-470/minocycline treated animals was greater than that in the untreated tumors. The addition of carbogen breathing for 1 hr. prior to and during radiation delivery further increased the radiation response so that overall there was a 2-fold increase in the tumor growth delay compared with untreated animals. Administration of the perflubron emulsion along with carbogen breathing prior to and during radiation delivery resulted in a 3-fold increase in tumor growth delay compared with that obtained with radiation alone. There was a linear relationship between decrease in the percent of pO_2 readings ≤ 5 mmHg and tumor growth delay at each radiation dose indicating that the diminution in tumor hypoxia produced by these treatments may be directly responsible for the increase in the effectiveness of the radiation therapy. [Supported by NIH grants P01-CA19589 and R01-CA50174.]

INTRODUCTION OF NEGATIVE CHARGE ON THE *IN VIVO* BEHAVIOR OF A HEMOGLOBIN CROSSLINKED WITH bis (3,5-dibromosalicyl) sebacate.

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Crosslinked hemoglobins (XLHb) do not dissociate into αß dimers, do not cross the glomerular filter and have a plasma half time (PHT) 3 to 4 times longer than unmodified hemoglobin. We have found that human hemoglobin reacted with bis (3,5-dibromosalicyl)sebacate (XLHb-DEC) forms a stable tetramer and has a PHT similar to that of other XLHbs i.e. 3.2 hours. This PHT is still less than that of albumin of similar molecular weight. The longer PHT for albumin may be a function of its larger negative charge acting to decrease its rate of passage across capillary endothelia (non glomerular). The present studies were done to determine if the addition of negative charges to XLHb-DEC would extend its PHT. XLHb-DEC, acylated with succinic anhydride, produced a molecule which by differential gel filtration appeared as a stable tetramer and by electrophoresis to have substantially increased negative charge. Plasma retention studies in anesthetized rats indicated the PHT of this derivative to be 3.5 hours. This value was not different from XLHb-DEC and both are less than the 5.2 hour PHT determined for albumin (evans blue method). It appears that introduction of negative charges did not increase PHT. However, larger urinary excretion of the succinylated species, as compared to the XLHb-DEC, suggests that 1. the introduction of negative charges may have led to *in vivo* instability of the tetramer which could not be detected by the biophysical methods employed or 2. the molecule had an effect to increase glomerular permeability.

UNMODIFIED STROMA FREE HEMOGLOBIN (SFH) IMPROVES BLOOD PRESSURE AND RENAL FUNCTION IN THE HYPOTENSIVE RAT: ROLE OF SFH-INDUCED NITRIC OXIDE (NO) INACTIVATION.

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We have compared the effects of an intravenous infusion of unmodified SFH and an isooncotic solution (serum albumin) on mean arterial blood pressure (MAP) and glomerular filtration rate (GFR) following induction of hypovolemic hypotension by acute hemorrhage in the anesthetized rat.

Protocol 1: MAP and GFR were measured before and after subjecting rats to hemorrhage (20ml blood/kg body weight over 10 minutes). Then, rats received 2ml of either unmodified SFH (17g%) or an isooncotic albumin solution (the oncotic pressure of both solutions was 92mmHg). MAP and GFR were again measured post-infusion.

	BASELINE	POST-HEMORRHAGE	POST- INFUSION
MAP (mmHg):			
Albumin	114 ± 2	63 ± 3*	82 ± 2*¶§
SFH group	110 ± 4	61 ± 6*	111 ± 7¶
GFR (ml/min)			
Albumin	3.0 ± 0.2	1.0 ± 0.2*	2.0 ± 0.2*¶§
SFH	2.8 ± 0.3	1.1 ± 0.5*	2.7 ± 0.3¶

*=p<0.05 vs. baseline; ¶=p<0.05 vs. post-hemorrhage period; §=p<0.05 vs. SFH group during the post-infusion period. (n=8 in each group).

Thus, MAP and GFR following hemorrhage were both increased to a substantially greater extent by unmodified SFH than by albumin

Protocol 2: The purpose of this protocol was to elucidate the role played by hemoglobin-induced NO inactivation in the effects of SFH on MAP. Rats were subjected to hemorrhage as described above. Then, rats received 2ml of either vehicle (Ringers lactate), albumin or unmodified SFH and MAP was determined. Then, the NO synthase inhibitor, L-NAME was administered (0.1mg/kg/min) while continuing infusion of vehicle, albumin or SFH,

	POST-HEMORRHAGE	POST-INFUSION	POST-LNAME
MAP (mmHg)			
Vehicle	60 ± 3	61 ± 2†	94 ± 8*¶
Albumin	63 ± 3	82 ± 4*†	104 ± 7*¶
SFH	66 ± 1	119 ± 3*†	112 ± 3*

*=p<0.05 vs post-hemorrhage period; †=p<0.05 vs other groups during post-infusion period; ¶=p<0.05 vs post-infusion period. (n=4 in each group)

Thus, NO inhibition increases MAP following both vehicle and albumin infusion but has no additional effect on MAP following infusion of SFH suggesting that the NO system is already maximally inhibited by SFH.

Conclusion: SFH raises MAP and GFR in the hypotensive, hypovolemic rat in part by expanding intravascular volume (via a colloid effect) and in part by peripheral vasoconstriction induced by inactivating NO.

REDISTRIBUTION OF TISSUE OXYGEN TENSION DURING PERFUSION WITH CROSS-LINKED HEMOGLOBIN IN AWAKE HAMSTERS DUE TO CHANGES IN VESSEL WALL pO_2 GRADIENTS

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The pO_2 oxygen gradient across the arteriolar vessel wall, defined by the difference between intravascular and tissue pO_2 immediately adjacent to the vessel, was assessed by the Pd-meso-tetra (carboxyphenyl) porphyrin phosphorescence decay technique in subcutaneous muscle of the awake hamster skinfold model. Tissue pO_2 was measured at locations distant from large vessels. Isovolemic/isooncotic hemodilution was performed with $\alpha\alpha$ cross-linked free hemoglobin solution [(3.5-bis(dibromosalicyl)fumarate), LAIR, San Francisco] to a hematocrit of 15%. Capillary flow velocity was not statistically different from control. Results are tabulated [C: Control; Hct: %; BP: blood pressure, mm Hg; * & ** indicate $p<0.05$ & $p<0.01$; pO_2, grad: gradient, mm Hg]

		Art., 57 ± 15 μm		Ven., 70 ± 21 μm		Tissue
	Hct/BP	PO_2	grad.	PO_2	grad.	PO_2
C	48/80	52±6	16±5	32±10	11±8	22±5
$\alpha\alpha$Hb	15/**104***	54±15	**27**±8*	18±10	5±3	**11**±5*

Microcirculatory oxygen carrying capacity (product of capillary flow velocity, potential blood oxygen content and functional capillary density, FCD) decreased by 45% from control due to decreased FCD. These findings indicate that vasoconstriction augments wall oxygen gradients and possibly metabolism, providing the energy for the increased arteriolar tone required in elevating blood pressure in the presence of significant reductions in blood viscosity. The redistribution of tissue pO_2 appears to be due to increased consumption of oxygen by the arteriolar vessel wall, shown by the notable increase of the pre-existing vessel wall gradient.
Supported by USPHS grant HL48018 and an Erwin-Schrödinger Fellowship J00912-MED[1].

ROLE OF NITRIC OXIDE SCAVENGING IN PERIPHERAL VASOCONSTRICTOR RESPONSE TO BOVINE $\beta\beta$ CROSSLINKED HEMOGLOBIN

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Transfusion with many crosslinked hemoglobin solutions causes an increase in arterial pressure that may be mediated by scavenging of nitric oxide (NO). If so, we postulated that inhibiting synthesis of NO after hemoglobin transfusion would fail to cause vasoconstriction ordinarily seen with such inhibition. In pentobarbital anesthetized cats, we tested whether administration of the NO synthase inhibitor, L-nitro-L-arginine methyl ester (L-NAME; 10 mg/kg, iv), produced peripheral vasoconstriction after isovolemic exchange transfusion with hemoglobin (n=8) to the same extent as occurs with L-NAME infusion in time controls (n=8), and in controls matched for reduced hematocrit (17%) with albumin transfusion (n=8). Bovine hemoglobin was treated aerobically with bis (3,5-dibromosalicyl) fumarate to produce $\beta\beta$-81 lysine crosslinks (P50=17 mmHg; n=1.8). Regional blood flow (ml/min/100g) was measured with radiolabeled microspheres. Hemoglobin exchange transfusion resulted in an increase in mean arterial pressure from 134 $\pm$ 8 to 166 $\pm$ 7 mmHg ($\pm$ SE) and there was no further increase after L-NAME. In contrast, L-NAME increased pressure in time controls (119 $\pm$ 12 to 149 $\pm$ 14 mmHg) and albumin controls (112 $\pm$ 6 to 144 $\pm$ 9 mmHg). Hemoglobin transfusion decreased intestinal (42 $\pm$ 5 to 25 $\pm$ 4) and renal (298 $\pm$ 21 to 164 $\pm$ 17) blood flow, and there was no further decrease after L-NAME. In contrast, L-NAME decreased blood flow to intestines in time controls (52 $\pm$ 8 to 31 $\pm$ 4) and albumin controls (61 $\pm$ 5 to 37 $\pm$ 7), and to kidneys in time controls (225 $\pm$ 20 to 118 $\pm$ 11) and albumin controls (293 $\pm$ 24 to 191 $\pm$ 19). We conclude that the lack of intestinal and renal vasoconstriction to L-NAME after hemoglobin transfusion is best explained by hemoglobin scavenging of NO prior to NO synthase inhibition. (Supported by NIH HL48517.)

EFFECT AND SAFETY OF LIPOSOME ENCAPSULATED HEMOGLOBIN "NEO RED CELLS (NRC)" AS A PERFUSATE FOR TOTAL CARDIOPULMONARY BYPASS

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We evaluated the effect of liposome encapsulated hemoglobin "Neo Red Cells (NRC)" as a priming solution for total cardiopulmonary bypass (TCPB) using a dog. The TCPB was started after removal of 57.1 - 73.7% of autologous blood and continued for 7 hours. During TCPB using NRC, the total peripheral resistance index (TPRI) decreased to 1/3 of TPRI when red blood cells (RBC) were used. This change suggests that NRC, the viscosity of which is lower than that of RBC, reduced the load on the circulation system. The oxygen volume delivered by NRC was higher than that delivered by RBC, resulting in a greater oxygen consumption with NRC. During TCPB using NRC, the serum LDH level was lower than that using RBC. Another experiment was to investigate the influence of NRC on the reticuloendotherial system. After injection of NRC, human hemoglobin, derived from the NRC, was detected in the spleen and liver. However, this hemoglobin was degraded and eliminated from spleen and liver within 7 days. The phagocytic index decreased temporarily immediately after NRC injection, but this improved within a day. We conclud that NRC is effective and safe as a perfusate for TCPB.

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ALTERATION OF CEREBRAL MICROCIRCULATION BY HEMODILUTION WITH HEMOSOME IN AWAKE RATS

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Our study showed that hemodilution with modified fluid gelatin resulted in an increase in local cerebral blood flow (LCBF), but no change at all in local cerebral oxygen delivery (LCOD) in rats. Hemosome, a lecithin encapsulated hemoglobin having the oxygen-carrying capacity, was developed to improve LCOD by hemodiluton. Therefore, we have hypothesized that LCBF & LCOD would be increased by hemodilution with hemosome . To test this hypothesis, adult male Sprague-Dawley rats weighing ~350g were used and divided into the hemodilution and the control groups. The rats were anesthetized with 1% isofluorothan for bilateral femoral arteries and veins catheterization. After the wounds were infiltrated with xylocaine and closed ,the rats were fixed onto lead bricks by putting casts around their hindlimbs and allowed for recovering from anesthesia. Hemosome was made from pig red blood cells and lecithin. It's mean diameter was ~0.5um and hemoglobin concentration was ~4g/dl. Isovolemic hemodilution, which lowered the systemic hematocrit from ~50% to ~30%, was achieved by rapidly replacing blood with the same volume of hemosome. Ten min later, LCBF in 14 brain structures were measured using the ^{14}C-iodoantipyrine technique. Our results showed that LCBF of the control group ranged from 114±17ml/100g/min in the medulla oblongata to 283±30ml/100g/min in the sensorimotor cortex. LCBFs in 13 out of the 14 structures were similar in the control and the hemodilution groups. However LCODs were generally decreased by 25% in the hemodilution group than the control group. In conclusion, hemodilution with hemosome does not improve LCBF & LCOD in rats.

EFFICACY OF RED CELL SUBSTITUTES

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A number of Red cell substitute candidates have now been tested in Phase I (safety) protocols. These, according to preliminary reports, have resulted in only minor side effects, including "flu-like" symptoms in recipients of perfluorocarbon- based products and mild elevations of blood pressure in recipients of hemoglobin-based products. Thus, it appears that attention can now be turned to the design of Phase II (efficacy) testing.

Efficacy is directly linked to the clinical indication for the product. In current practice, red cells are transfused primarily to replace blood lost in surgery or trauma. However, since volume can be replaced by safe non-oxygen-carrying plasma expanders which are already approved for clinical use, the question is whether it is possible to demonstrate first that red cell substitutes actually deliver oxygen to tissues, and second, that there is some therapeutic benefit from doing so. The first is relatively simple, the second very complex.

Normally, oxygen bound to hemoglobin within the red cell must first dissociate from the protein, then pass through a cascade of diffusion steps before reaching mitochondria where it is reduced by terminal oxidases in oxidative phosphorylation. Some of these steps may represent rate-limiting barriers to diffusion, and others may not. When red cells are replaced by extracellular oxygen carriers, whether based on hemoglobin or perfluorocarbons, the diffusion cascade will be altered, perhaps in significant ways. These alterations should be understood in order to take full advantage of the potential clinical applications for the new products.

Since the goal of transfusion therapy is to prevent tissue ischemia, rather than to treat it, there may be no objective markers for successful treatment either with blood or a blood substitute. We must therefore consider the use of "surrogate" end points, such as physiological markers, in efficacy testing.

Possible physiologic markers for adequacy of oxygen supply to tissues include oxygen uptake, fractional oxygen extraction, and mixed venous oxygen tension. Although it may not be possible to convincingly demonstrate efficacy using these measures, they can be used to show that a product in question actually delivers oxygen to tissue. The larger question of patient benefit may be answered only by the demonstration of reduced use of allogeneic blood or by overall improved clinical outcomes such as hospital stay, wound healing, infection rates, or other surgical complications.

DOUBLE CROSSLINKED AND MULTILINKED HEMOGLOBINS AS POTENTIAL BLOOD SUBSTITUTES

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Double crosslinking, in which two different reagents are used or the same reagent is under two different conditions, and multilinking, in which a reagent that reacts at more than two sites in used, offer the possibility of greater control over the stability and function of a hemoglobin-based blood substitute. Oxy and deoxy hemoglobin crosslinked by DFDNB (difluorodinitrobenzene) gave only monomers and crosslinked dimers on SDS gels. Denaturation gave two transitions with T_m's of 6 and 20°C. This crosslinker can span 3Å, locking the quaternary structure more tightly and give greater thermal stability than longer reagents. Three double crosslinked hemoglobins have been produced. The first was made by crosslinking purified ß82-fumarate crosslinked Hb with dimethylpimelimidate. It had a T_m of 63°C, compared to 57°C for the singly crosslinked proteins. Thus, double crosslinking only slightly increased the stability over that of singly crosslinked hemoglobins. Double crosslinking experiments using fumarate and DFDNB crosslinking gave similar results. A more interesting double crosslinked hemoglobin has fumarate crosslinks between both the 99 lysines and the ß82 lysines. The T_m is 20°C, which is 4°C greater than the singly crosslinked species. The oxygen affinity and cooperativity are similar to that of 99XLHb but unaffected by inositol hexaphosphate (IHP). The autoxidation is slower than that of 99XLHb. (Supported in part by a grant from the Research Corporation.)

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